IN VITRO AND FUNCTIONAL ANALYSIS OF THE *ARABIDOPSIS ADH* 5' FLANKING SEQUENCE

BY

WILLIAM LEE MCKENDREE, JR.

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WILLIAM LEE MCKENDREE, JR.

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The *Adh* (*alcohol dehydrogenase*) gene of *Arabidopsis* is expressed constitutively in immature seedlings and cells in suspension, and may be induced in roots of mature plants. In vivo DMS footprinting of the 5' flanking region of this gene has identified sites of protein-DNA interaction which include the G-box element also found in several light regulated genes (RbcS, Cab, and Chs). The *Adh* G-box binds specifically to a protein component of *Arabidopsis* extracts from cell culture and mature leaves, and mutations of footprinted G-box bases partially or completely disrupt binding. Corresponding G-box mutations in the full length *Adh* promoter resulted in >60% reduction in reporter gene activity when assayed in *Arabidopsis* protoplasts. *Adh* promoter mediated expression was observed in both 4-day-old and 15-day old *Arabi-*

dopsis seedlings transformed by microprojectile bombardment. Deletions of the *Adh* promoter were assayed in *Arabidopsis* seedlings by particle bombardment and in *Arabidopsis* protoplasts. Sequence domains which are necessary for wild type gene activity were confined to first 390 bp 5' to the transcription start site and included all sites of in vivo protein-DNA interaction previously identified by Ferl and Laughner (1989). Deletion of 5' sequences from -390 to -289 decreased activity by 50%, and further deletion to -177 resulted in a decrease to less than 10% of full gene activity.

CHAPTER 1
INTRODUCTION

Terrestrial green plants keep their photosynthetic structures more or less fixed in space and are therefore faced with the need to adapt to a variety of environmental stresses, as opposed to simply relocating to a more favorable environment. Those plant species more able to sense and respond to the stresses of their environment are therefore selected through evolution. This unique feature of green plants has provided a variety of model systems for the inquiry of molecular bases for stress adaptation.

Of the many hazards which befall the stationary organism, flooding is one of the most life threatening. Molecular oxygen concentrations in water preclude aerobic metabolism for the organ of concern, and alternate biochemical pathways which do not require oxygen are needed for the flooding duration. Early work into the basis of this flooding response in *Zea mays* (Hageman and Flesher, 1960) led to the identification of the alcohol dehydrogenase (ADH, E.C. 1.1.1.1.) enzymes (Schwartz and Endo, 1966; Schwartz, 1966) and eventually to the cloning of genes which encode them (Gerlach et al., 1982; Dennis et al., 1984). ADH isozymes regenerate NAD^+, thus allowing

glycolysis to continue in the absence of terminal oxidation. Anaerobic ATP production is adequate to allow the plant to survive limited periods of stress.

The means by which these stress induced genes are regulated with regard to activity and cell type will be the subject of this investigation. ADH is expressed in cell types possessing elevated rates of metabolic activity in addition to oxygen deprived cells of the mature root. ADH activity has been observed in a number of higher plants including wheat, soybean, beet, rice, pea, barley, tomato, tobacco, corn, pearl millet, and *Arabidopsis* (from Dolferus and Jacobs, 1991). Expression patterns of *Adh* vary slightly among those plant species investigated, yet retain an overall similarity which reflects *Adh* function(s).

The expression patterns of *Adh* in *Zea mays* and *Arabidopsis thaliana* will be summarized briefly. This will be followed by a review of 5' flanking sequence-mediated mRNA synthesis and a comparison of *Adh* transcriptional regulation among relevant genes and organisms. Emphasis will be placed on protein/DNA interactions within specific 5' flanking sequences and their apparent ability to mediate cell-type specific, developmentally regulated and inducible expression of *Adh*.

ADH Expression Characteristics

Zea mays

ADH activity in corn arises from a two-member gene family, *Adh*1 and *Adh*2. ADH enzymatic activity resulting from one or both genes is found in the root, scutellum, embryo, aleurone, endosperm, pollen, stem pith and nodes, and the etiolated leaf (Bailey-Serres et al., 1987), as well as cells in suspension (Paul and Ferl, 1991). The level of ADH expression in roots, scutellum, immature endosperm, and embryo increases under anaerobic stress. *Adh*1 and *Adh*2 differ in cell-type specific expression and in their degree of inducibility (Bailey-Serres et al., 1988; Paul and Ferl, 1991). In general, ADH is active in tissues with high rates of metabolic activity or where oxygen is in limited supply.

Adh1 and *Adh2* are found on chromosomes 1 and 4 respectively (Schwartz, 1971; Dlouhy, 1979). The two genes are 82% similar in coding DNA and 87% similar in amino acid sequence (Dennis et al., 1984; Dennis et al., 1985). The *Adh* genes encode a 379 aa protein (Dennis et al., 1985), with the functional ADH enzyme being a dimer of one or both gene products (Freeling and Schwartz, 1973). Both 5' and 3' untranslated regions of the maize *Adh* genes differ considerably in nucleotide sequence (Dennis, 1984; Dennis, 1985). However, some short regions of 5' untranslated sequence are similar between

*Adh*1 and *Adh*2 and will be considered in a following section.

Anaerobically induced *Adh* activity arises from an increase in *Adh* mRNA transcription (Gerlach et al., 1982; Vayda and Freeling, 1986), eventually reaching 20 to 50 times that found in uninduced roots (Ferl et al., 1980; Gerlach et al., 1982; Rowland and Strommer, 1986; Paul and Ferl, 1990),. Anoxia is followed by the selective translation of 20 proteins, including ADH (Sachs et al., 1980). Once oxygen stress is relieved, *Adh* message is rapidly depleted (Rowland and Strommer, 1986).

The two *Adh* genes differ slightly in their developmental expression patterns in that *Adh2* is not detectable in pollen (Bailey-Serres et al., 1987), whereas *Adh1* is abundant. They are, however, transcribed at roughly equivalent levels in the cob, silk, leaf, node, stem, and uninduced root of the mature plant (Paul and Ferl, 1990). *Adh* mRNA levels in the root increase tenfold or more following 8 hours of anaerobic induction, and *Adh2* mRNA is present at approximately twice the level of *Adh1*. This pattern is also observed for cells in suspension, where *Adh2* mRNA increases to roughly three times that of *Adh1* mRNA under similar induction conditions (Paul and Ferl, 1990).

The rate of *Adh* mRNA synthesis may be regulated by protein/DNA interactions within the 5' flanking sequences of the *Adh* coding region (Ferl and Nick, 1987; Walker et

al., 1987; Chen et al., 1987; also, see review by DeLisle and Ferl, 1990). Deletion or mutation of this region of the *Adh*1 gene results in reduced expression of a reporter gene when assayed in protoplasts for transient expression (Howard et al., 1987; Walker et al., 1987; Lee et al., 1987) or in sunflower tumors for stable expression (Ingersoll, 1990). A discussion of these and other related studies will follow.

Regulation of tissue specific expression of *Adh* by its 5' flanking sequence has not been conclusively demonstrated in maize, and dicot species transformed with *Adh* via Agrobacterium have given poor results (Ellis et al., 1987). Indirect evidence supporting tissue specific regulation by maize *Adh1* 5' flanking sequence does exist, however, from an unstable, organ-specific *Adh*1 mutant isolated from a Robertson (Robertson, 1978) mutator line (Chen et al., 1987). In this mutant, *Adh1-3F1124*, ADH1 is expressed at roughly 6% normal levels in seed and anaerobically treated seedling, but at normal levels in pollen. A *Mu 1* transposable element insertion was found 31 bp 5' to the TATA element and creates a potential new TATA element. 5' sequence mediated tissue specificity for *Adh* expression is probable (but not proven) in that other tissue-specific plant genes such as *RbcS* are known to possess this property (Nagy et al., 1985).

Adh expression studies have been conducted in other monocot plants, namely rice (Xie and Wu, 1989), barley

(Hanson and Brown, 1984; Hanson et al., 1984; Trick et al., 1988), Wheat (Susseelan and Bathia, 1982), and pearl millet (Bannuett-Bourrillon, 1982). In each of the above mentioned species, *Adh* is encoded by two or three genes, activity is induced with anoxia and expression is regulated in a developmental and tissue specific manner.

One notable exception in *Adh* tissue specific expression occurs in rice. *Adh* activity may be induced in mature roots, embryos, endosperm, etiolated and green mature leaves (Xie and Wu, 1989). Although green leaves show relatively low levels of *Adh* activity following anaerobic induction, this and all other *Adh* activity continues to increase for up to 96 hours. All other *Adh* expression characteristics for rice are similar to corn, including transcriptional regulation and induction of activity by 2,4-D (Xie and Wu, 1989; Freeling, 1973).

Arabidopsis thaliana

Of the several dicot species in which *Adh* has been characterized, *Arabidopsis* has proven to offer the widest range of possibilities for analysis of expression both in vitro and in the whole plant (Meyerowitz and Pruitt, 1985). *Arabidopsis* has been the system of choice for gene expression studies due to its small habit and genome size (70,000 kb)(Leutwiler et al, 1984; Meterowitz and Pruitt, 1985). Plants may be grown by the thousands in a

single flat and will produce seed 4 to 6 weeks following germination. *Arabidopsis* is susceptible to Agrobacterium mediated transformation (Valvekens et al., 1988; Feldman, 1991) and has been used to characterize the expression of a number of plant genes. ADH activity in *Arabidopsis* is observed in germinating seeds, young seedling cotyledons and roots (<10 DAP), the anaerobically induced mature root (>10 DAP), in the stigma and in pollen grains (Dolferus and Jacobs, 1985; Dolferus and Jacobs, 1990). As in corn, ADH activity is not observed in green plant parts except in the vicinity of vascular bundles in stems, leaves and roots (Dolferus and Jacobs, 1990). Specific ADH activity in young seedlings is highest 3 days following germination and decreases to undetectable levels by day 10 (Dolferus and Jacobs, 1985).

ADH activity may be induced in the mature seedling and callus tissue using either anoxia or the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Anaerobic induction of ADH activity in 10- to 14-day-old seedlings results in a 10- to 15-fold increase in *Adh* mRNA (Dolferus and Jacobs, 1985), versus the 20 to 50 fold increase observed in corn. Poly (A^+) mRNA isolated from induced callus will direct the synthesis of ADH in an in vitro translation system (Dolferus and Jacobs, 1985). Induction of ADH activity with 2,4-D has not been observed, however, for cells in suspension (Dolferus and Jacobs, 1985).

Arabidopsis possesses only one *Adh* gene (Chang and Meyerowitz, 1986), as opposed to two for corn and three for barley (Trick et al., 1988). This gene encodes a protein identical in length and 81% similar in amino acid sequence to maize *Adh1*. Arabidopsis ADH forms an active heterodimer with maize ADH when extracts from the two species are mixed (Fisher and Schwartz, 1973). Furthermore, antibodies raised against maize ADH cross-react with *Arabidopsis* ADH (Dolferus and Jacobs, 1991). The *Arabidopsis Adh* gene is 73% similar in nucleotide coding sequence to maize *Adh*1. *Arabidopsis* Adh possesses 6 introns, compared to the 9 found in maize *Adh*, and these are found in the same positions as their counterparts in maize. Introns IV, V, and VII of maize *Adh* are not present in *Arabidopsis Adh* (Dolferus and Jacobs, 1990).

Comparison of 5' untranslated regions of the maize and *Arabidopsis Adh* genes reveals a disappointing lack of significant sequence homology. There are, however, small elements of sequence homology which have been investigated for potential regulatory function in this and other gene systems (Ferl, 1990) and whose investigation will be continued in this study.

Regulation of mRNA Transcription

Several plant gene systems are now available for the study of mRNA synthesis, as regulated by 5' flanking sequences, and their subsequent developmental and/or organ specific expression. This discussion will be

limited to those gene systems which share common putative regulatory elements or characteristics with maize or *Arabidopsis Adh*.

5' flanking DNA of maize *Adh*

Initial characterization of maize *Adh*1 regulation fell into two major categories: 1) Examination of protein/DNA interactions within the 5' flanking sequences by in-vivo DMS footprinting (Church and Gilbert, 1984; Ferl and Nick, 1987) and DNase I or restriction endonuclease hypersensitivity (Paul et al., 1987; Ferl, 1985) and 2) Expression of reporter genes regulated by various domains (deletions) of the *Adh*1 5' flanking region (Howard et al., 1987; Lee et al., 1987; Walker et al., 1987). Virtually all current molecular studies in corn *Adh*1 are derived from this experimental foundation.

In vivo DMS footprinting of the maize *Adh*1 promoter (Ferl and Nick, 1987) reveals roughly five domains of putative protein/DNA interaction. Cells in suspension were either aerated normally or anaerobically induced prior to DMS guanosine methylation and subsequent genomic sequencing (Church and Gilbert, 1984). Interactions fell into two general categories, those which are present regardless of induction state (-110, -120, -130) and those which appear in only anaerobically induced cell suspensions (-95, -180, -130). As will be discussed

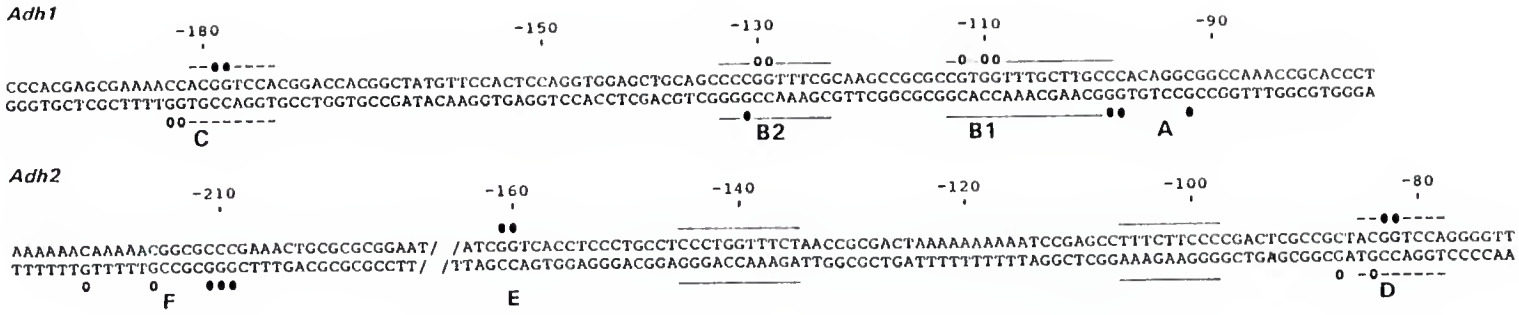
later, many of these regions have since been shown to bind protein in vitro and play a role in regulated expression. In vivo footprinting of maize *Adh*2 (Paul and Ferl,1990) reveals putative sites of protein/DNA interaction as well. Sites of interaction appear in three general regions. A footprint is observed, however, at position -85 which is strikingly similar in pattern and recognition sequence to that found at position -180 in maize *Adh*1 (Figure 1-1). The two footprints are labeled "C' (*Adh*1) and "D" (*Adh*2) and are induced by anaerobic stress. The possible involvement of these sites in regulation has not as yet been tested functionally.

In situ digestion of DNA within intact nuclei using either restriction endonucleases or DNase-I has been used to further characterize protein/DNA complexes of *Adh*1 (Ferl, 1985, Paul et al., 1987). Using restriction endonuclease Pst 1, a significant alteration in enzyme access is observed at -147 following anaerobic induction. Other regions examined were 5', at -417 and -1104, where corresponding in vivo DMS footprinting data are not available.

DNase-I hypersensitivity is an alternate method of characterizing the accessibility of chromatin associated with *Adh* transcription. Maize cells in suspension, either aerobic or anaerobically induced, were used for isolation of nuclei and in situ DNase-I digestion (Paul

Figure 1-1. *In Vivo* DMS Footpring of Maize *Adh1* and *Adh2*.

The 5' flanking sequences of maize *Adh1* and *Adh2* are shown, along with locations of observed *in vivo* DMS footprinting. Closed circles indicate protection, and open circles indicate enhancement. From Paul, A-L, and Ferl, R. J.(1990).


Adh1
-180
-150
-130
-110
-90
CCCACGAGCGAAAACCACGGTCCACGGACCACGGCTATGTTCCACTCCAGGTGGAGCTGCAGCCCCGGTTTCGCAAGCCGCGCCGTGGTTTGCTTGCCCACAGGCGGCCAAACCGCACCCT
GGGTGCTCGCTTTTGGTGCCAGGTGCCTGGTGCCGATACAAGGTGAGGTCCACCTCGACGTCGGGGCCAAAGCGTTCGGCGCGGCACCAAACGAACGGGTGTCCGCCGGTTTGGCGTGGGA
C
B2
B1
A
Adh2
-210
-160
-140
-120
-100
-80
AAAAAACAAAAACGGCGCCCGAAACTGCGCGCGGAAT/ /ATCGGTCACCTCCCTGCCTCCCTGGTTTCTAACCGCGACTAAAAAAAAAAATCCGAGCCTTTCTTCCCCGACTCGCCGCTACGGTCCAGGGGTT
TTTTTTGTTTTTGCCGCGGGCTTTGACGCGCGCCTT/ /TTAGCCAGTGGAGGGACGGAGGGACCAAAGATTGGCGCTGATTTTTTTTTTAGGCTCGGAAAGAAGGGGCTGAGCGGCGATGCCAGGTCCCCAA
F
E
D

et al., 1987). Regions of hypersensitivity were then classified constitutive (-160 to -700) or inducible (-35 to -150). These results agree well with those obtained from the two previous methods with regard to anaerobic induction, and may be used to delimit anaerobic responsiveness to within 200 bp of the transcription start.

Functional analyses; transient

Specific 5' flanking sequences of maize *Adh* have been shown to direct the anaerobically induced expression of a reporter gene in transformed maize protoplasts (Howard et al., 1987). Functional analysis of 5' deletions of the full length (1.1 kb) promoter shows that full activity and anaerobic inducibility are retained from -1094 to -140 (Walker et al., 1987). Inducible expression is still observed following deletion to -124, but this resulted in a significant (>50%) decrease in activity. Linker scanning analysis through the -133 to -72 region (Walker et al, 1987) identified two distinct regions necessary for anaerobic inducibility at approximately -130 and -105. When either of these Anaerobic Response Elements (AREs) are disrupted by DNA replacement, there is a complete loss in anaerobic induction.

Lee et al. (1987) conducted a functional analysis of the Adh 5' flanking region of maize as well, and obtained results supporting Walker et al. (1987). Large segments of the *Adh* promoter were removed by restriction

digest so that "deletions" corresponded to restriction enzyme recognition sequence locations. The full length promoter (1.1 kb) extended to the BamH1 site and was assigned 100% activity. Deletion to the Xba1 site (-417) resulted in a reduction to 65% of full activity, and further deletion to Pst1 (-147) reduces activity to 18%. The question of anaerobic inducibility was not addressed here.

The results obtained by Lee et al. (1987) are in agreement with those from Walker et al. (1987) in that 147 bp of 5' flanking *Adh* sequence is sufficient for the expression of a reporter gene. What remains in question is the degree to which activity is reduced by the various deletions. This matter is clarified by the work of Ingersoll (1990) as described below.

Functional analyses; stable transformation

An alternative method for functional analysis of 5' flanking sequences is the stable transformation of plant tissue using *Agrobacterium*. Briefly, the sequence element of interest is cloned into a plasmid carrying the desired reporter gene, a reference gene if desired, and a selectable marker. This plasmid is then introduced into the Agrobacterium host which is then used to infect plant tissue. If the appropriate sequence elements and virulence genes are present, the desired DNA sequences will

be transferred to the plant and be incorporated into the plant chromosome. The chromosomal site of incorporation varies among transformation events, so several transformed plants are needed for each construct to provide statistical accuracy. This method has been successfully applied to dicot species, but has had limited success in monocots.

Regulation of tissue specific expression of a gene is best analyzed in the transgenic plant. This method has been employed to dissect the well characterized cauliflower mosaic virus (CaMV) 35S promoter (Benfey and Chua, 1990). This promoter is known to confer constitutive expression on a reporter gene in both transient and stable expression systems (Odell et al., 1985; Jefferson et al., 1987). It was found that specific subdomains acted in a modular fashion to confer expression of the reporter gene on various organs (Benfey and Chua, 1990). Furthermore, the manner in which the subdomains functioned was dependent on the species in which they were expressed. Their results emphasize the need for caution when interpreting the tissue specificity of promoter mediated expression within a heterologous genetic background.

Ingersoll (1990) has functionally analyzed the maize 5' flanking sequence in plant tumors derived from sunflower. This method has the advantage of allowing for the analysis of larger amounts of transformed tissue than the

protoplast transformation affords, thus allowing for the direct quantitation of test and reference RNA. This work confirmed the location of the ARE(s) in maize *Adh*1 to the region between -140 and -100. A loss of 70% of full activity resulted by deleting to -140, and this deletion preserved anaerobic inducibility. These results are in agreement with those obtained by Walker et al.(1987) and Lee et al. (1987).

A convincing functional analysis of maize *Adh* in the mature transgenic plant has not been accomplished. The maize plant is refractory to Agrobacterium transformation, and maize *Adh* introns are not properly processed in dicot species (Ingersoll, 1990). Ellis et al. (1987) attempted to analyze maize *Adh*1 5' flanking DNA in transgenic tobacco. Although *Adh* 5' flanking sequences were able to confer anaerobic inducibility, this required the addition of non-*Adh* regulatory sequences (CAMV 35S) to assist in overall transcription levels.

Many of the 5' flanking sequence elements of *Adh*1 which have been identified by one of the methods discussed above have been subjected to in-vitro analysis of protein binding by the gel retardation assay (Fried and Crothers, 1981). Gel retardation, or the mobility shift assay, is performed by incubating a radioactive DNA fragment with the protein extract of interest, which is followed by fractionation by non-denaturing electrophoresis. This method of characterization allows for the

identification of the type(s) and amounts of protein involved in a regulatory "domain." These proteins may then be purified and used to obtain a clone of the gene which encodes them. Such information is essential for the elucidation of pathways governing development and stress response.

In general, 5' flanking regions which produce an in vivo DMS footprint appear to operate as functional units when the results of deletion studies are used for comparison. Putative regulatory sequence elements in maize *Adh*1 are characterized by 1) the C-rich domain found at -130 and 2) the GTGG motif at -98, -110, and -180 (Paul and Ferl, 1990). This is also true for maize *Adh*2, with a C-rich footprint at -210, and GTGG elements footprinting at -160 and -80 (Paul and Ferl, 1990).

An oligonucleotide resembling the maize *Adh*1 -130 region was constructed for analysis of in-vitro protein binding (Ferl, 1990). A component of crude whole cell extracts from maize suspension cultures was found to bind specifically to this sequence. Further analysis of the -130 binding protein (ARF-B2) shows that it is a multicomponent complex which binds the -130 element regardless of the state of induction, supporting previous results from in vivo DMS footprinting (Ferl and Nick, 1987). Attempts to clone the gene encoding ARF-B2 and other maize *Adh*1 regulatory genes are in progress.

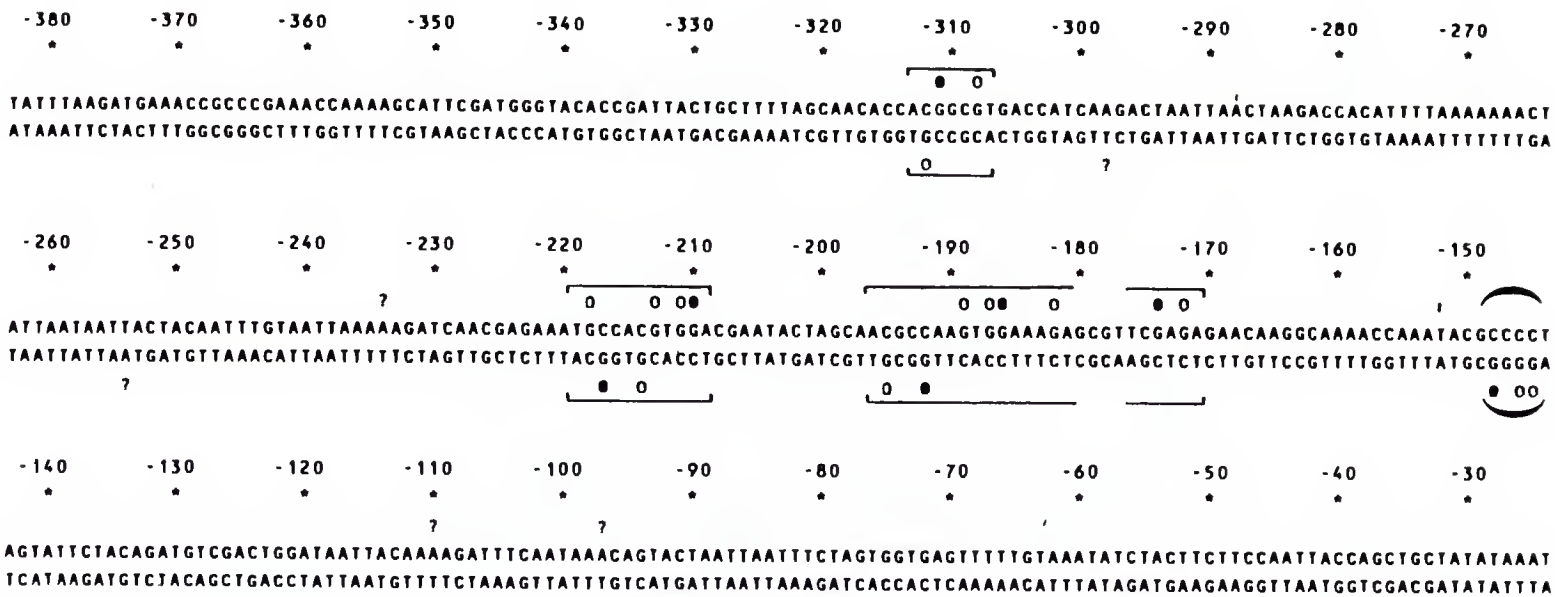
5' flanking DNA of *Arabidopsis Adh*

As with maize *Adh*, the in-vivo DMS footprint of *Arabidopsis Adh* has revealed several sites of protein/DNA interaction (Ferl and Laughner, 1989). Footprints are observed at positions -145, -175, -190, -210, and -310 (Figure 1-2). The DNA sequence elements at these locations are similar to those found in maize in that they are also either 1) C-rich (-145), or 2) have GTGG nearby (-190, -210, and -310). The element which footprints at position -210 is unique in that it possesses a perfect GTGG dyad, and will be discussed in greater detail in a following section. The element which footprints at position -175 has no obvious similarity to footprinting elements in maize *Adh*.

The dyad sequence element which footprints at position -210 had been identified in a number of plant gene 5' flanking sequences, and has been termed the G-box (Giuliano et al., 1988). The G-box element is present in the 5' flanking sequence of ribulose-1,5-bisphosphate carboxylase small subunit (RbcS) genes of several dicot species, including *Arabidopsis* (figure 1-3). It is also present in the promoters of several chalcone synthase (CHS) genes, the patatin gene from *Solanum*, and the rolbc gene of Nicotiana (Schulze-Lefert et al., 1989).

Figure 1-2. *In Vivo* DMS footprinting of the *Arabidopsis Adh* 5' Flanking Sequence.

Regions of *in vivo* footprinting are shown in brackets. The G-box element is shown at -210. Curved brackets at -145 show the region of maize *Adh1* ARE homology. Protection interactions are depicted by closed circles, and enhancements by open circles. From Ferl, R. J., and Laughner, B. H. (1989).


-380 -370 -360 -350 -340 -330 -320 -310 -300 -290 -280 -270
TATTTAAGATGAAACCGCCCGAAACCAAAAGCATTCGATGGGTACACCGATTACTGCTTTTAGCAACACCACGGCGTGACCATCAAGACTAATTAACTAAGACCACATTTTAAAAAAACT
ATAAATTCTACTTTGGCGGGCTTTGGTTTTCGTAAGCTACCCATGTGGCTAATGACGAAAATCGTTGTGGTGCCGCACTGGTAGTTCTGATTAATTGATTCTGGTGTAAAATTTTTTTGA
-260 -250 -240 -230 -220 -210 -200 -190 -180 -170 -160 -150
ATTAATAATTACTACAATTTGTAATTAAAAAGATCAACGAGAAATGCCACGTGGACGAATACTAGCAACGCCAAGTGGAAAGAGCGTTCGAGAGAACAAGGCAAAACCAAATACGCCCCT
TAATTATTAATGATGTTAAACATTAATTTTTCTAGTTGCTCTTTACGGTGCACCTGCTTATGATCGTTGCGGTTCACCTTTCTCGCAAGCTCTCTTGTTCCGTTTTGGTTTATGCGGGGA
-140 -130 -120 -110 -100 -90 -80 -70 -60 -50 -40 -30
AGTATTCTACAGATGTCGACTGGATAATTACAAAAGATTTCAATAAACAGTACTAATTAATTTCTAGTGGTGAGTTTTTGTAAATATCTACTTCTTCCAATTACCAGCTGCTATATAAAT
TCATAAGATGTCTACAGCTGACCTATTAATGTTTTCTAAAGTTATTTGTCATGATTAATTAAAGATCACCACTCAAAAACATTTATAGATGAAGAAGGTTAATGGTCGACGATATATTTA

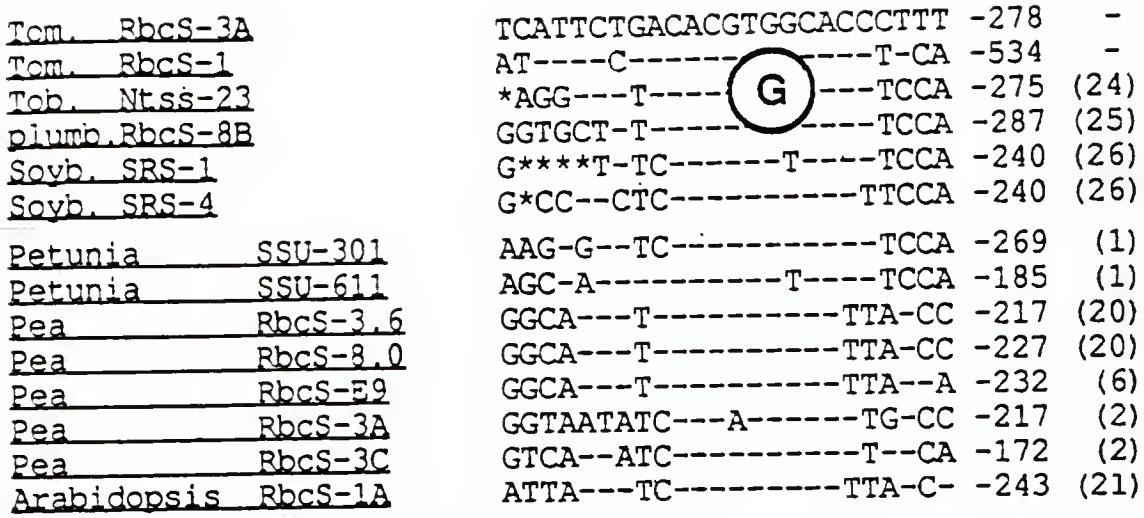


Figure 1-3. Comparison of RbcS G-box elements.

The sequence elements shown above are from 5' Flanking sequence of selected RbcS genes. The areas of G-box homology are shown by the circled G to the right. the abbreviations shown are as follows. Tom.=tomato, plumb.=tobacco, and soyb.=soybean. Numbers in parentheses to the right are reference numbers in the original manuscript. From Giuliano et al., (1989).

RbcS 5' flanking sequence

Members of the RbcS gene family, like those of the *Adh* gene family, are environmentally inducible and expressed in a cell-type specific manner (Tobin and Silverthorne, 1985). RbcS mRNA is induced in the presence of light primarily in leaf and stem tissue and is not detectable in roots (Sugita et al., 1987). RBCS activity is, in part, regulated at the transcriptional level (Tobin and Silverthorne, 1985; Kuhlemeir et al., 1987).

The RbcS gene family has been comparatively well characterized at the molecular level. Comparison of 5' flanking sequences from several RbcS genes has identified three general elements, of sequence homology (Kuhlemeier et al., 1987; Giuliano et al., 1988); 1) the G-box, C/A-CACGTGGC (Giuliano et al., 1988), 2) the I box, which has the consensus sequence 5'-GATAAG-3'(Giuliano et al, 1988), and 3) the GT box, which has a very broad consensus sequence (Green et al, 1988; Kuhlemeir et al., 1988).

In order to demonstrate protein binding to these putative regulatory elements, 5' flanking sequence fragments from several RbcS genes were examined using the gel retardation assay (Giuliano et al., 1988). Nuclear extracts from tomato and *Arabidopsis* were found to contain a protein component which binds specifically to upstream RbcS sequences from tomato, pea, and *Arabidopsis*.

This result was further verified by performing DNase I footprinting on the protein/DNA complex (Giuliano .op et al, 1988). In this method, the DNA sequences of interest are either bound to protein or naked, subjected to partial digestion by DNase1, and fractionated by denaturing electrophoresis. Regions which are "protected" from digestion show a "footprint" as compared to naked DNA. These footprints correspond roughly to the site of protein binding.

Footprinting of RbcS 5' flanking sequences, in conjunction with gel retardation results, verified the region of specific protein binding to be the G-box (Giuliano et al, 1988). In contrast, DNase1 hypersensitivity was detected in the region of the I box. Footprinting of the GT box (Giuliano et al, 1988) failed to reveal interactions with those sequence elements from either tomato or *Arabidopsis* crude extract.

The GT box consensus and I box consensus for the Rbcs gene family does not obviously align with regions of in-vivo DMS footprinting in either maize or *Arabidopsis Adh*. However, the GT box of a few Rbcs genes (Kuhlemeier et al., 1987) resembles the GTGG observed in maize and *Arabidopsis Adh* (-310, -190) and is homologous to the SV40 core type II element (Zenke et al., 1986).

Functional analysis of the 5' flanking sequences of Rbcs genes in transgenic plants has provided the bulk of

information available regarding sequence elements necessary for gene activity and photoactivation. Initial characterization delimited a 352 bp deletion mutant of the pea rbcS-E9 gene which retained photoinducibility and tissue specificity (Nagy et al., 1985). Deletion of RbcS-E9 or RbcS-3A (Fluhr et al., 1986) confirmed the results of Nagy et al. (1985) and further defined sequences necessary for organ specificity and photoinducibility to within 240 bp 5' of transcription start.

More recently, Kuhlemeier et al. (1989) demonstrated that a region from -50 to +15 of the RbcS-3A promoter was able to confer light responsiveness, but not organ specificity, on a reporter gene in transgenic plants. Furthermore, it was demonstrated that this promoter element interacts with an upstream element between -189 and -156 to allow high level expression. This region contains no previously described element of homology. A region from -150 to -230 does, however, produce an in-vitro footprint (Green et al., 1987), and includes the G-box (-217) and binds the G-box factor (Giuliano et al., 1988). It is suggested that this domain (-189 to -156) may contain a novel regulatory element. It should be noted here that their constructs contained additional enhancer elements (35S, soybean heat shock element) foreign to the gene of interest in order to boost expression levels.

Further experiments designed to precisely define thelocation of the light responsive element(s) and the function of the G-box were conducted on the *Arabidopsis* rbcS-1A gene in transgenic tobacco (Donald and Cashmore, 1990; Donald et al., 1990). This work defined a 196 bp region from -320 to -125 which was sufficient to confer light regulated, tissue specific expression on an *Adh* reporter gene. This region contains GT, G, and I boxes. Mutations or deletions affecting GT box(es) had no significant effect on expression. Disruption of the I box resulted in a significant decrease in reporter gene activity, and mutations or deletions which disrupted the G-box abolished all reporter gene activity.

The presence of the G-box element in other light regulated genes has led to an association of this element with light responsiveness. In addition to functional analyses of promoter function in the RbcS gene family, the chalcone synthase (CHS) and chlorophyll a/b binding protein (CAB) genes have also contributed to the understanding of the G-box and other putative 5' regulatory elements.

CHS and CAB 5'flanking sequence

The *in vivo* DMS footprint has not been successfully conducted on an RbcS gene. Footprint data are available, however, for the Chs gene of parsley (Schulze-Lefert et al., 1989). CHS activity is inducible in parsley suspen-

sion cultures by UV light, and activation occurs at the transcriptional level (Chappel and Hohlbrock, 1984).

Three sequence domains of the Chs promoter produce an in-vivo DMS footprint upon UV irradiation of parsley cells in suspension. These domains (labeled regions I, II, and III) occur at positions -140, -165, and -235. Region III resembles the GT box of RbcS genes, and, when assayed for function in a transient expression, appears to function as a transcriptional enhancer (Schulze-Lefert et al., 1989). Region II is homologous to the G-box of Rbcs and *Arabidopsis* genes, and footprints in a manner nearly identical to that of the *Arabidopsis* G-box. When region II is deleted or mutated for functional analysis (Schulze-Lefert, 1989), there is a nearly complete (>90%) loss of reporter gene activity, as well as a loss in photoinducibility. It should be noted here, however, that this deletion also included region I, and region I deletion or mutation results in an equally severe reduction in reporter gene activity.

In order to more carefully determine the structure of putative cis-acting elements, site directed mutagenesis was employed to mutate and change spacing between these elements (Block et al., 1990). These constructs were then assayed for transient expression in parsley protoplasts. Their results defined a 7 base core sequence critical for G- box activity. When the distance

between box I (as previously defined by Schulze-Lefert et al.,(1988)) and the G-box was increased by 4 bp, reporter gene activity was essentially abolished. However,deleting the space between the G-box and box III had no significant effect. It is proposed that the G-box functions in close association with at least one other regulatory interaction.

This result is in agreement with those obtained for 5' flanking sequences of the RbcS gene family. Regulatory interactions of 5' flanking sequences do not appear to function with complete independence, in spite of their modularity.

Summary

Plants may respond to environmental stress by modification of gene expression. The perception of changes in atmospheric oxygen, light, temperature, and water availability results in a modification of the type of genes transcribed and translated. The modes of signal transduction for these plant responses remain unknown. In the case of plant Adh genes, mRNA transcription is accelerated following anoxia, and rates of mRNA turnover are low. Gene activity is therefore increased by induction of mRNA transcription.

Induction of gene activity is correlated with protein binding to 5' flanking sequences of that gene. The in vivo footprint of the maize Adh1 promoter changes following anoxia. Sequences of the promoter which footprint in vivo, and bind protein in vitro, are contained within regulatory promoter elements as determined by deletion or mutation analysis.

One of these regulatory elements, the G-box, has been found in genes of diverse function. Genes which contain this element are expressed in a wide variety of tissue types and are regulated by mechanisms which are not obviously similar. Regulatory elements appear to function with considerable modularity, and this G-box element has apparently been recruited by many genes for an as yet undetermined purpose.

Sites of protein-DNA interaction revealed by in vivo footprinting of Arabidopsis Adh will be characterized for in vitro protein binding. The Adh 5' flanking sequence will then be subjected to functional analysis in transformed Arabidopsis. Critical regulatory elements will be identified and a model of Arabidopsis Adh transcriptional activation will be proposed.

CHAPTER 2
CHARACTERIZATION OF PROTEIN INTERACTIONS WITH *Arabidopsis Adh* 5' FLANKING SEQUENCE

Introduction

Transcriptional regulation of gene expression involves the interaction of DNA binding proteins with the gene of interest (Dynan et al.,1985), and localization of these sites of interaction has been facilitated by the technique of *in vivo* dimeththylsulfate (DMS) footprinting (Church and Gilbert,1984; Nick and Gilbert, 1985). The *in vivo* DMS footprints of the maize *Adh1* and *Arabidopsis Adh* genes have revealed several sites of putative regulatory interaction which are similar in sequence between these two genes, namely, the C rich box and a moderately conserved 5'-GTGG-3' motif (Ferl and Nick, 1987; Ferl and Laughner, 1989). However, only in the *Arabidopsis Adh* gene (at position -210) is this GTGG motif present on both strands as a perfect dyad (Ferl and Laughner, 1989). This symmetrical element (5'-CCACGTGG-3') has also been identified in the 5' flanking sequence of *ribulose-1,5-bisphosphate carboxylase* small subunit (*RbcS*) genes of several species and is termed the G-box (Giuliano et al., 1988).

Expression of the *alcohol dehydrogenase* (*Adh*) and *ribulose-1,5-bisphosphate* (*RbcS*) genes of higher plants is both cell-type specific and environmentally inducible, yet the tissues in which they are expressed, their modes of induction, and their protein functions are quite distinct. *RbcS* mRNA is induced in the presence of light primarily in leaf and stem tissue, and is not detectable in roots (Sugita and Gruissem, 1987).

The G-box of tomato *RbcS-3A* has been shown to bind specifically to protein from crude nuclear extracts from tomato and Arabidopsis by the gel retardation assay and G-box elements from other *RbcS* genes, including *Arabidopsis RbcS-1A*, compete specifically for G-box protein binding (Giuliano et al., 1988).

In this chapter it will be demonstrated from the *in vitro* binding competition assay (Fried and Crothers, 1981) and from both *in vivo* and *in vitro* DMS footprinting (Church and Gilbert, 1984; Treisman, 1986; Ferl and Nick, 1987) that a protein component of *Arabidopsis* whole cell extract from cultured cells and mature leaves binds the *Arabidopsis Adh* G-box in a manner comparable to that observed in cultured cells *in vivo* (Ferl and Laughner, 1989). Comparison of *in vivo* G-box binding in cultured cells and leaves, however, reveals significant differences between these tissue types, and that *in vivo Adh* G-box binding in leaves is very weak or does not occur.

Materials and Methods

Gel Retardation Assay

Whole cell extracts were prepared by variations of Manley et al. (1980) and Wu (1984). Cell cultures or leaves were frozen in liquid nitrogen before homogenization at 4°C in 15 mM Hepes, pH 7.6, 40 mM KCl, 5.0 mM MgCl, 1.0 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). One-tenth volume of 4.0 M ammonium sul fate was added and the slurry centrifuged for 30 min at 19,000 g, 4°C. To the supernatant, 0.3 g/ml ammonium sulfate was added and mixed on ice for 60 min. Protein was then precipitated at 15,000g for 20 min., 4°C. This pellet was resuspended (1.0 ml/10 g tissue) in 20 mM Hepes, pH7.6, 40 mM KCl, 1.0 mM DTT, 0.5 mM PMSF, 0.1 mM EDTA, and 10% glycerol), then dialyzed 4 hr in two changes of 100 volumes of 20 mM Hepes, pH7.6, 40 mM KCl, 0.1 mM PMSF, 0.1 mM EDTA, 10% glycerol, and 5 mM -mercaptoethanol. After dialysis the extract was frozen with liquid nitrogen and stored at -80°C until use. Binding reactions were performed at room temperature in 13 ul containing 10 ul (60 ug) of crude extract. All binding reactions contained either 1 ul (50 ng) competitor DNA, or extract buffer as the no competitor control, one ul of end-labeled *Adh* G-box oligonucleotide (1.0 ng), and 1.0 ul of 1.0 M KCl (unless otherwise specified) for a final

KCl concentration of 110 mM. A 5 minute pre-binding of competitor DNA and protein was followed by addition of labeled oligonucleotide probe, a 5 minute additional incubation, and electrophoresis on a 5% non-denaturing 89 mM Tris-Cl, 89 mM Boric acid, and 2.6 mM EDTA polyacrylamide gel at 30 mA.

In Vitro DMS Footprinting

In vitro DMS footprinting was carried out as described (Treisman, 1986). Following electrophoretic fractionation of binding reactions or probe alone, gel slices were electroeluted, cleaved at methylated G residues with piperidine, and fractionated by electrophoresis on a 10% polyacrylamide-urea gel. Approximately equal counts were loaded per lane. The extent of methylation protection varies among G residues within a given footprint and is highly reproducible.

UV Crosslinking

Modification of the cross-linking procedure of Chodosh et al. (1986) was used for DNA probe synthesis and binding reactions. Probe was synthesized by first annealing a specific primer to the 3' end of the top strand of the -210 oligonucleotide. The second strand

was synthesized using Klenow fragment in the presence of 25 ng of DNA template, 25 uM dGTP, 50 uM 5-bromo-2'deoxyuridine triphosphate (Sigma), and 50 uCi each of $-^{32}$P-dATP and $-^{32}$P-dCTP. Binding reactions are essentially identical to the gel retardation binding reactions with the following exception that reactions contain approximately 1ng labeled oligonucleotide probe. Binding is followed by 15 min exposure to UV light, nuclease digestion and SDS gel electrophoresis (12.5 %). The gel was fixed, dried, and autoradiographed for 48 hr.

RESULTS

The G-box binds a protein component of *Arabidopsis* crude extract

Oligonucleotides corresponding to previously determined sites of *in vivo* protein-DNA interaction at positions -310, -210, and -140 of the *Arabidopsis* 5' flanking region (Ferl and Laughner,1989) were synthesized. The -210 oligonucleotide was utilized as the probe for all in vitro gel retardation and DMS footprinting experiments. Figure 2-1 shows the *Arabidopsis Adh* oligonucleotides along with those corresponding to the G-box of *Arabidopsis RbcS-1A* (Krebbers et al., 1988), and the G-box like sequence of the adenovirus major late promoter (MLP)

Competitor DNA		Sequence	
-140	-149	GCCCCTAGTATTCTGC	-134
-310	-318	ACACCACGGCGTGACCAT	-301
-210	-222	GAATGCCACGTGGACTGCA	-204
RG	-264	ATCTTCCACGTGGCATTA	-247
MLP	-63	GGCCACGTGACC	-52

Figure 2-1. Oligonucleotides used for gel retardation assay.

Sequences of the Arabidopsis Adh 5' flanking region about which are observed in vivo DMS footprinting (Ferl and Laughner, 1989) were used to design oligonucleotides for the gel retardation assay. The -210 oligo (Adh G-box) is used for all experiments as the probe. The RbcS G-box oligonucleotide (RG) was synthesized from the -243 region of Arabidopsis RbcS-1A (Krebbers et al., 1989). The MLP oligonucleotide corresponds to the -60 region of the MLP of Adenovirus (Chodosh et al., 1989). Only the top strand of the sequence is shown. All probe and competitor DNA is in >90% double stranded form.

enhancer (Chodosh et al., 1989). The MLP synthesized is identical in length and sequence to that presented by Chodosh et al. (1989) to be sufficient for specific binding to a component of yeast crude extract, and was chosen as a representative of G-box like mammalian enhancer element for this experiment.

Subjecting crude whole cell extract (Manley et al, 1980) from Arabidopsis cell cultures to the gel retardation assay (Fried and Crothers, 1981) revealed two distinct types of G-box binding activity, Forms I and II (Figure 2-2). A third less distinct form is observed which migrates more rapidly than Forms I or II. Lane 1 shows bound and free forms resulting in the absence of competitor DNA. Lanes 2 through 7 represent binding reactions carried out in the presence of increasing amounts of the heterologous competitor DNA, poly(dI-dC). The addition of as little as 10 ng of poly(dI-dC) significantly increases the intensity of Form II, with a corresponding reduction in intensity of Form I (lane 2). Continued increase in poly(dI-dC) to 500 ng (lanes 3,4,5, and 6) resulted in the reduction and eventual elimination (lane 6) of Form I. Form II, however, increased in intensity up to 500 ng. Further increase of poly(dI-dC) to 1.0 ug prevented formation of Form I and significantly reduced the intensity of Form II.

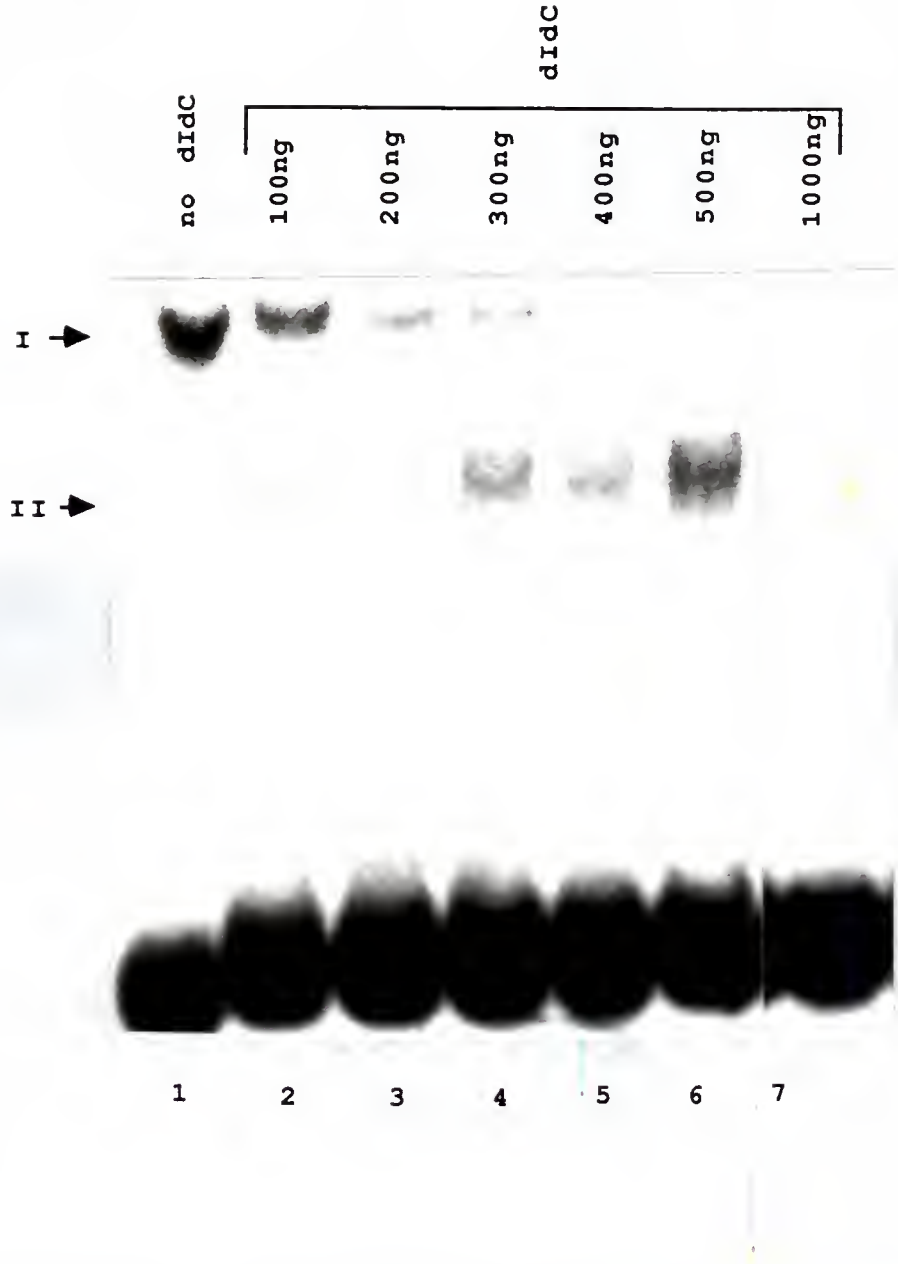


Figure 2-2. Gel retardation assay of Arabidopsis crude extract binding to the Adh B-box (-210).

Crude whole cell extract from Arabidopsis cells in suspension is bound to end-labeled, double stranded -210 probe for gel retardation assay (Fried and Crothers, 1981). Binding reactions were carried out in the absence of competitor DNA (lane 1), or in the presence of poly-(dIdC) at 100 ng (lane 2), 200 ng (lane 3), 300 ng (lane 4), 400 ng (lane 5), 500 ng (lane 6), and 1.0 ug (lane 7).

In order to examine the influence of ionic strength on binding affinity of Forms I and II, a retardation assay was conducted with cell suspension crude extract in the presence of increasing amounts of KCl (Figure 2-3, A). Stringent binding conditions were maintained by the addition of 1.0 ug poly(dI-dC) to each reaction. Lane 1 shows a binding reaction identical to that in lane 7 of Figure 2-2, with KCl at 40 mM. Increasing KCl concentration to 100 mM results in a marked increase in binding affinity of protein to the -210 probe. Continued increase of KCl to 200 mM greatly reduced the amount of Form II, and binding in the presence of 300 mM essentially precludes binding to the -210 probe.

To demonstrate that retarded electrophoretic species are due to protein binding, a binding reaction was carried out in the presence of proteinase k (Figure 2-3, B). Lane one shows a typical Form I retarded complex using 1.0 ug poly(dIdC) competitor DNA. Form I is not present when binding is carried out in the presence of proteinase k (lane 2).

G-box protein binding specificity was determined by competition analysis (Figure 2-4). Form I binding to the Adh G-box appears to be nonspecific, as it is observed in the absence of competitor DNA (lane 1), in the

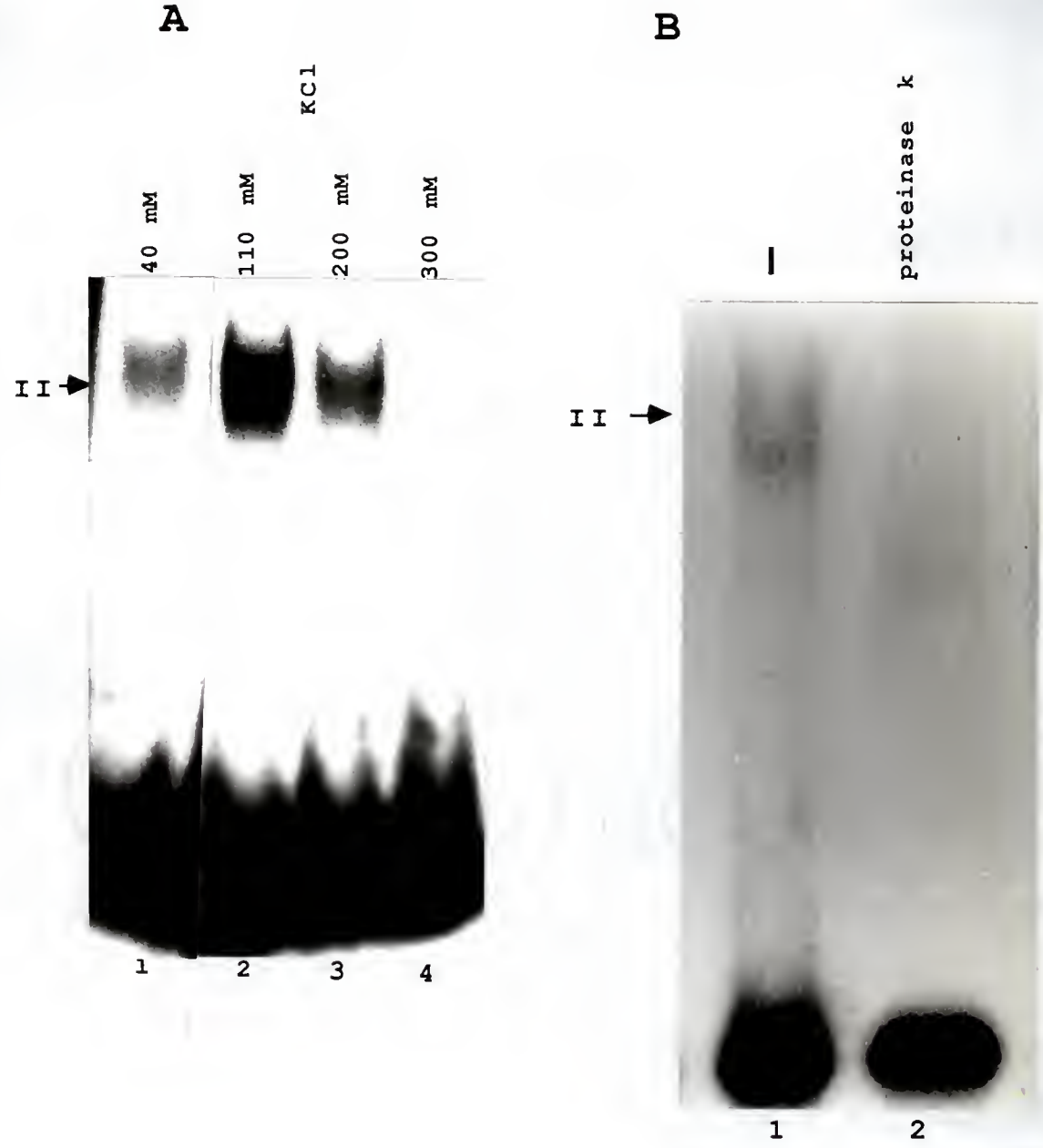


Figure 2-3. Influence of KCl concentration on G-box binding affinity.

A) Binding reactions of the Adh G-box (-210) probe with Arabidopsis crude whole cell extract were conducted in the presence of 1.0 ug poly-(dIdC), and KCl at 40 mM (lane 1), 100 mM (lane 2), 200 mM (lane 3), 300 mM (lane 4).

B) Binding reactions were carried out as in lane 1 above (lane 1), or in the presence of proetinase k (lane 2).

presence of 50 ng of both heterologous and homologous competitor oligonucleotides (lanes3 to 7), but not in the presence of 1.0 ug of poly(dI-dC)(lane 2). Form II persists in the presence of up to 1.0 ug poly(dI-dC)(lane 2) as well as 50 ng of heterologous competitor DNA (lanes 3, 4, and 7), yet is obliterated by 50 ng of homologous competitor Adh and RbcS G-boxes (lanes 5 and 6). There fore, Form II appears specific for protein binding to both the Adh and RbcS G-boxes. The G-box like MLP sequence is not effective in competition for G-box binding.

The results of competition analysis obtained with leaf extract are similar to those from cell culture extract (Figure 2-4, lanes 8-14) with two exceptions. Form IV persists in the presence of all competitor DNA (lanes 9 to 14), suggesting that it is nonspecific Adh G-box binding. The specific binding activity from leaf extract comparable with the Form II observed using cell culture extract is represented by Form III, a protein-DNA complex that was slightly reduced in migration rate relative to Form II.

Competition experiments were also conducted in the presence of 1.0 ug of poly(dI-dC) to demonstrate selective binding in excess heterologous carrier DNA. The results of this experiment are shown in Figure 2-5. Binding to the G-box -210 probe in cell culture and leaf extract was conducted in the absence (lanes 1 and 6) or

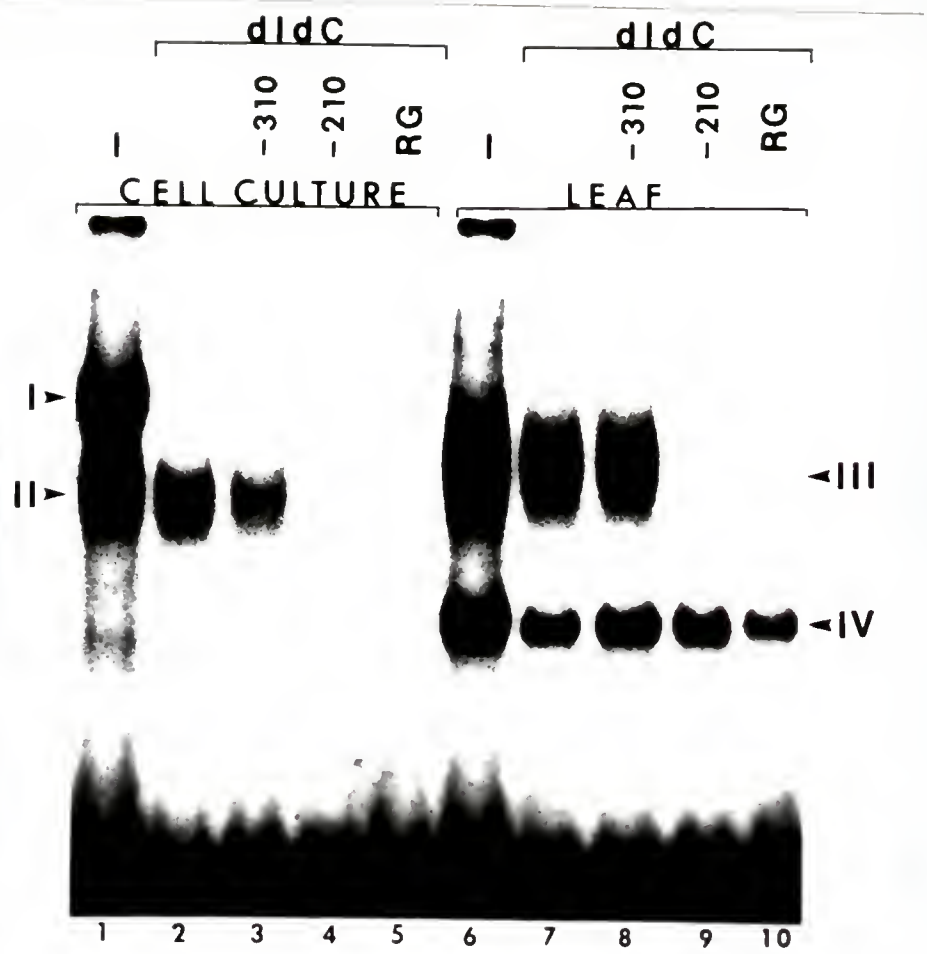


Figure 2-4. Adh G-box (-210) Binding Factor Is Found in Both Cell Culture and Leaves of Arabidopsis.

Crude whole cell extracts from cells in culture (lanes 1 to 7) or mature leaf tissue (lanes 8 to 14) were assayed for Adh G-box binding competition. Binding reactions were carried out either in the absence of competitor DNA (lanes 1 and 8), or in the presence of 1.0 ug of poly(dIdC)(lanes 2 and 9) or 50 ng of the following oligonucleotides: -310 (lanes 3 and 10), -140 (lanes 4 and 11), -210 Adh G-box (cold probe)(lanes 5 and 12), RG (lanes 6 and 13), or MLP (lanes 7 and 14).

presence (lanes 2 to 5, and 7 to 10) of 1.0 ug of poly(dI-dC) in addition to 50 ng of the following oligonucleotide competitors: -310 (lanes 3 and 8), -210 (lanes 4 and 9), and RG (lanes 5 and 10).

Form I of the cell culture extract binding reaction is present in lane 1 and absent in lanes 2 and 5. As was seen in the previous experiment, poly(dI-dC) competitor at 1.0 ug abolishes Form I [as will 1.0 ug of the -210 G-box oligonucleotide (data not shown)]. Form II, however, persists in the presence of either 1.0 ug poly(dI-dC) alone (lane 2) or 1.0 ug poly(dI-dC) plus 50 ng of -310 (lane 3), yet is abolished in the presence of 1.0 ug poly(dI-dC) plus 50 ng of -210 (lane 4) or 50 ng of RG (lane 5). These results are entirely consistent with the previous experiment (Figure 2-4), and are in support of Form II being a protein-DNA complex that is specific for G-box binding.

Results obtained using leaf extract in the presence of high amounts of poly(dI-dC) (Figure 2-5, lanes 6 to 10) are also consistent with the previous experiment. Bound Form III is observed in the absence of competitor DNA (lane 6), in the presence of 1.0 ug of poly(dI-dC) (lane 7), and in the presence of 1.0 ug poly(dI-dC) plus 50 ng of -310 (lane 8). Form III is abolished, however, when 1.0 ug of poly(dI-dC) and 50 ng of homologous competitor oligonucleotide are supplied to the binding

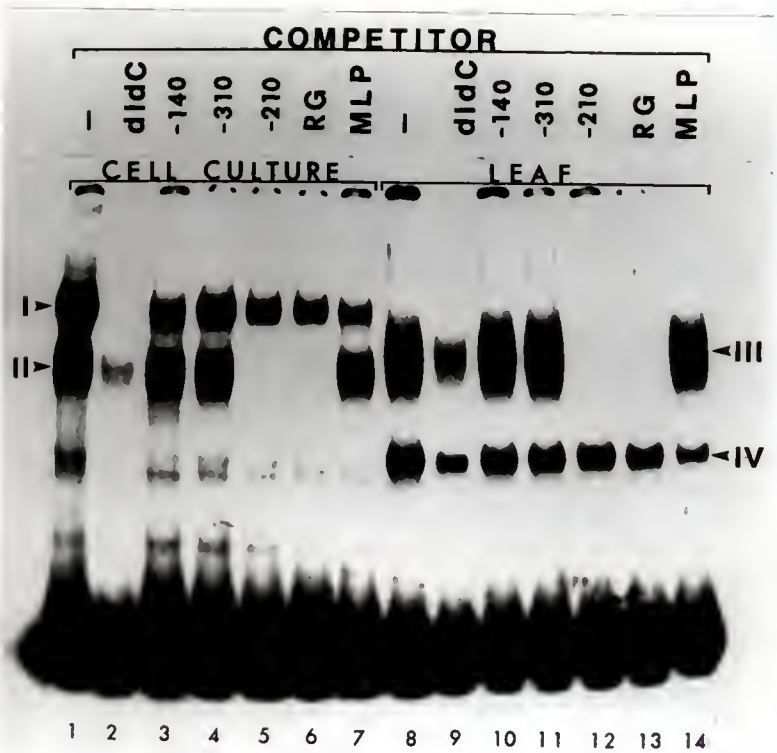


Figure 2-5. Binding specificity of Arabidopsis Crude extract is preserved in the presence of poly(dIdC).

Crude whole cell extracts from cells in culture (lanes 1 to 5) or mature leaf tissue (lanes 6 to 10) were assayed as in Figure 2-4, with modification of competitive binding conditions. Binding reactions were carried out either in the absence of competitor DNA (lanes 1 and 6), or in the presence of 1.0 ug poly(dIdC) alone (lanes 2 and 7), or in the presence of 1.0 ug of poly(dIdC) plus 50 ng of one of the following oligonucleotide competitor DNAs: -310 (lanes 3 and 8), -210 (lanes 4 and 9), and RG (lanes 5 and 10).

reaction. Form IV, again, persists in the presence of all competitor DNA. Therefore, it is concluded from these results that bound Form III represents a protein/DNA interaction of a specificity equivalent to that observed for bound form II.

Protein-DNA crosslinking

UV crosslinking experiments were conducted for determination of the molecular weight of protein(s) binding to the G-box oligonucleotide. Binding reactions are essentially identical to those performed for gel retardation experiments, and included 1.0 ug of poly(dI-dC) as heterologous competitor. No distinct bands are observed in the no protein control (Figure 2-6, lane 1). One predominant band of approximately 33 kd is observed when protein is included in the binding reaction (lane 2).

Binding specificity was tested by the addition of either -310 or -210 oligonucleotides. Competition with the -310 oligonucleotide is not observed at 20 ng (lane 2) or 200 ng (lane 3), but is effective at 2.0 ug (lane 4) in eliminating both electrophoretic forms. The corresponding analysis using -210 as competitor DNA is shown in lanes 5 (20 ng), 6 (200 ng), and 7 (2.0 ug).

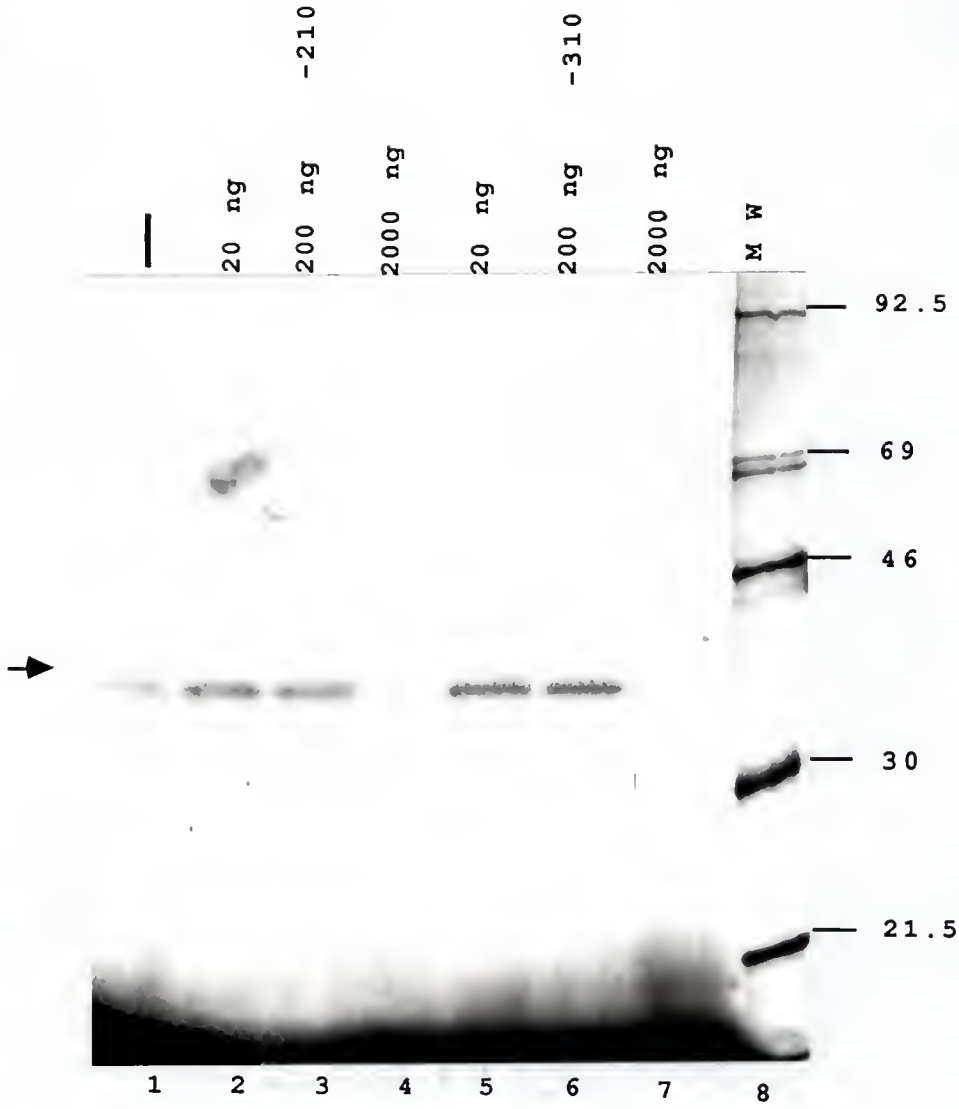


Figure 2-6. Protein-DNA Crosslinking of Arabidopsis Crude Extract with the Adh G-box (-210) Probe.

The Adh G-box probe was synthesized incorporating 5-Brdu for UV mediated protein-DNA crosslinking. Two electrophoretic forms, a conspicuous band at 55 kd and a faint band at 35 kd, are observed when binding is carried out in the absence of competitor DNA (lane 1). Binding reactions were also carried out in the presence of homologous competitor Adh G-box (cold -210) at 20 ng (lane 2), 200 ng (lane 3), or 2.0 ug (lane 4). Reactions were also performed in the presence of heterologous -310 competitor DNA at 20 ng (lane 5), 200 ng (lane 6), or 2.0 ug (lane 7).

Again, competition is observed only in the presence of 2.0 ug of -210, and is equal for both bands. A faint band is detectable in lane 5 at 55 kd, however, where one is not observed in lane 8.

In Vitro DMS Footprinting of the Adh G-box with Cell Culture and Leaf Crude Extract

The *in vitro* competition analysis (Figure 2-4) demonstrates equal specificity of protein factor G-box binding from cell cultures and leaves, but it does not indicate whether the protein-DNA interactions giving rise to equivalent specificity are identical. An *in vitro* DMS footprint (Treisman, 1986) of the *Adh* G-box complexes from cell culture and leaf crude extract (Forms II and III) is shown in figure 2-7.

Quantitative variation in the extent of methylation protection *in vitro* may be observed among G residues within either strand from both cell culture and leaf extract (lanes 3,6,9,12). Moderate protection is detected for G residues at positions -210 and -211 (cell culture) of the top strand and -217 of the bottom strand, whereas complete protection is evident for positions -213 and -218 of the top strand and -216 and -214 of the bottom strand. (The probe used for this assay was modified by the addition of PstI linkers at both the 5' and

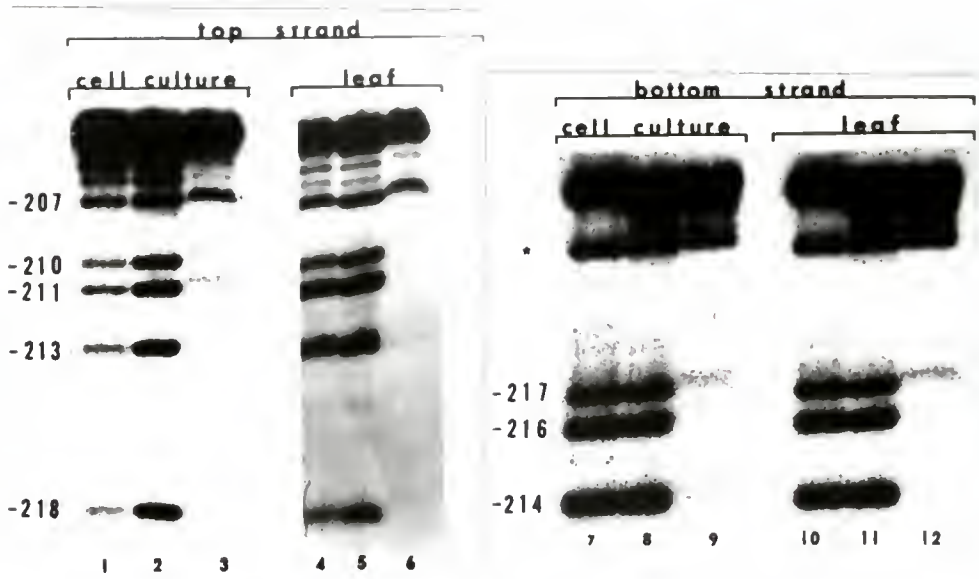


Figure 2-7. In vitro DMS footprinting of the Arabidopsis Adh G-box from Cell Culture and Leaf Whole Cell Crude Extract.

The -210 Adh G-box (Figure 2-1) double stranded oligonucleotide containing PstI ends was 5'-end-labeled on either the top strand only (left upper panel) or lower strand only (right lower panel). After binding with cell culture or leaf extract, the binding reaction was treated with DMS and subjected to preparative electrophoresis on gels identical to those used for the gel retardation assay. The bands corresponding to the free, unbound probe (lanes 2, 5, 8, and 11), bound form II (lanes 3 and 9), and bound Form III (lanes 6 and 12) were recovered by electroelution. As an additional control, the probes were treated with DMS in binding buffer without extract (lanes 1, 4, 7, and 10). The asterisk indicates a guanine residue outside the G-box, within the PstI end. This G-box oligonucleotide with PstI ends has identical bandshifting and competition qualities as the -210 oligonucleotide without PstI ends.

3' ends. The G residue of the bottom strand, indicated by a star in figure 3, is part of that linker sequence). These results agree well with those obtained by in vivo DMS footprinting of cell cultures (Figure 2-8).

Discussion

The G-box sequence is highly conserved among *RbcS* genes (Giuliano et al., 1988) and some other higher plant genes (Ferl and Nick, 1987; Ferl and Laughner, 1989; Schulze-Lefert et al., 1989). *in vivo* DMS footprinting of an *RbcS* gene is not yet available for comparison with that of *Adh*, but *in vivo* studies conducted in parsley for the *chalcone synthase* 5' flanking sequence have revealed a G-box protein interaction virtually identical to that observed in *Arabidopsis Adh* in cell culture (Schulze-Lefert et al., 1989).

Although G-box sequences have been identified in several *RbcS* and other plant genes, the only *Adh* gene known to contains the conserved G-box element is that from *Arabidopsis*. Maize *Adh1* and *Adh2* do not have dyad G-box elements (Gerlach, 1982; Dennis et al., 1985; Ferl and Nick; 1987). Comparison of factor binding sites in *Arabidopsis* and maize by *in vivo* DMS footprinting (Ferl and Laughner, 1989) shows that the conserved 4C box and a GTGG motif that is essentially half of a G-box are bound

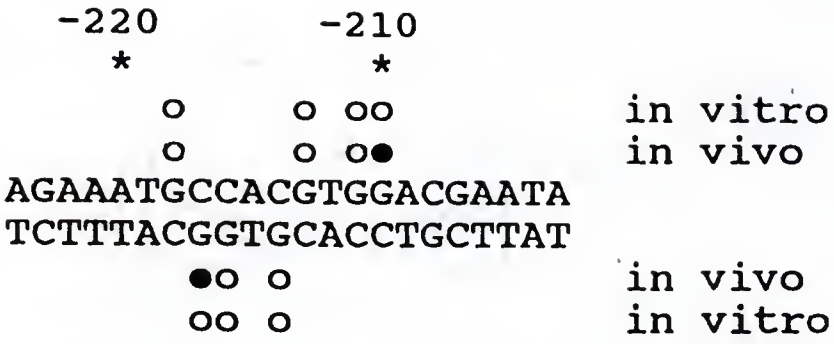


Figure 2-8. A Summary Comparison of in Vivo and in Vitro DMS Footprinting for the Arabidopsis Adh G-box.

Protein-DNA interactions are designated by circles. Open circles denote G residues protected from DMS methylation and solid circles indicate enhanced methylation.

in the 5' flanking sequence of both genes. We have found that these sequences do not compete for protein binding to the G-box *in vitro* (data not shown), and therefore suggest that these two element types are not bound by a G-box factor *in vivo*. The G-box factor is, therefore, separate and distinct from the factor(s) involved with the 4-C box or GTGG motifs.

RbcS sequences containing the G-box confer both organ specific and photoinducible expression in transgenic plants (Fluhr et al.,1986; Kuhlemeier et al., 1987). However, recent reports (Kuhlemeier et al., 1989; Ueda et al., 1989) have shown that regions containing the G-box are functioning as transcriptional enhancers, and that the G-box itself is not essential for photoactivation. G-box like sequences of animal promoters (Chodosh et al., 1989) are known to function as enhancer elements, but our binding competition results indicate that the MLP sequence either does not bind the same protein as the *Adh* G-box, or does so with a greatly reduced affinity.

It is interesting to note that the slight variation in electrophoretic mobility of the bound G-box between cell culture and leaf extract of *Arabidopsis* (figure 2-4) has also been observed between light grown and dark adapted tomato leaf extract (Giuliano et al., 1988). Extracts from dark adapted leaf tissue, in which *RbcS* genes are not induced, exhibited G-box binding which was

slightly more rapid in migration rate than that from light-grown plants, and this corresponds directly to what we observe, with cell culture complex ex (Form II) migrating more rapidly than that from leaves (Form III). In contrast, binding specificity and in vitro DMS footprinting of cell culture and leaf extract are striking in similarity, with the only notable difference being the top strand footprint at nucleotide -211. In combination, these results suggest that the G-box factor (GBF) may undergo cell-type specific modification or interaction with additional regulatory proteins. This is further supported by the observation of ADH activity in dark adapted leaves of maize (Bailey-Serres, 1987).

The differences we have observed in DMS modification of G residues between the in vivo and in vitro footprints, although possibly a result of the artificial nature of the in vitro binding environment, may be a result of examination of the G-box in the absence of adjacent protein-DNA interactions (Ferl and Laughner). This possibility may be addressed by increasing the length of the probe used for the in vitro DMS footprint to include other 5' flanking sequences which bind other protein factors in vivo. This would more closely resemble the in vivo binding conditions, and may assist in characterizing other putative regulatory interactions which were identified by the in vivo DMS footprint.

Mature Arabidopsis leaf tissue has also been subjected to *in vivo* DMS footprinting (A-L. Paul, personal communication), and is markedly dissimilar to that observed for cells in suspensinsion. It is therefore suggested that the *in vivo* difference between cell culture and leaf tissue in *Adh* G-box binding is not due to G-box factor structure, and may be due to protein-protein interactions or some cell-type variation in DNA modification or chromatin structure. The ability of *Adh* and *RbcS-1A* of *Arabidopsis* (and perhaps other genes with related G-boxes such as *chalcone synthase*) to utilize the same putative regulatory protein suggests a general role for the G-box which varies with both cell type and target gene in the induction of gene transcription in higher plants.

CHAPTER III
FUNCTIONAL ANALYSIS OF THE 5' FLANKING REGION OF *Arabidopsis Adh*

In contrast to the extensive analysis which maize *Adh* 1 5' flanking DNA has been subjected to, relatively little is known about the function of potential regulatory sequence elements of other *Adh* genes. Of the *Adh* genes which have been cloned to date, only *Arabidopsis Adh* has been characterized with regard to promoter function. Tobacco protoplasts were transformed (Dolferus and Jacobs, 1990) with constructs containing either the full 960 bp of *Arabidopsis Adh* 5' flanking DNA or a fragment which extended to -230, and subsequently assayed for reporter gene expression. Anaerobic induction was observed for full length promoter fused to both CAT and GUS reporter genes, whereas the truncated promoter allowed constitutive expression in the absence of oxygen. At tempts to transform *Arabidopsis* protoplasts were unsuccessful.

Dolferus and Jacobs (1990) also report a functional analysis of the truncated (-230bp) *Arabidopsis Adh* promoter fragment in transgenic *Arabidopsis*. CAT

activity was observed in root and shoot tissue, but not in flowers. Full length promoter constructs were not included in this study. These results are not in agreement with those obtained using tobacco protoplasts. Obviously, a genuine analysis of *Adh* regulation in *Arabidopsis* must include transient (and stable) expression characteristics of the *Adh* 5' flanking sequence in a homologous genetic background (ie. *Arabidopsis*).

Several methods are now available for the analysis of transient expression of reporter gene systems in *Arabidopsis* tissue, yet none have been successfully employed to date. *Arabidopsis* protoplast preparation and transformation has been conducted using a PEG/$Ca(NO_3)_2$ method for the purpose of regenerating transgenic plants from transformed protoplasts (Damm et al, 1988). However, transformation of *Arabidopsis* protoplasts by this method, or other methods such as electroporation, for the analysis of transient gene expression has not been reported.

Transformation by particle bombardment (Klein et al., 1988a; Klein et al., 1988b;) has been successfully employed in the transformation of cells in suspension and intact plants, including *Arabidopsis* (Bruce, et al.,1989; Seki et al, 1991). Particle bombardment requires far less DNA (<10 ug) than the conventional methods of electroporation or PEG incubation, and is not restricted to

any particular tissue or cell type.

Finally, plant cells or tissue may be transformed by silicon carbide fiber ("whiskers") injection (Kaeppler et al., 1990). Whiskers are suspended in water and mixed with the tissue of interest in a buffered solution containing DNA. The slurry is then vortexed briefly for fiber injection. This method is being used routinely for transformation of fly embryos (Cockburn, personal communication) and has been successful in transforming various tissues of the maize kernel (J. Baier, personal communication).

In this chapter I report the transformation of *Arabidopsis* for the functional analysis of the *Arabidopsis Adh* 5' flanking sequence. Deletions and mutations of the *Adh* 5' flanking sequence are compared to the native -950 bp fragment for regulation of transient expression of the GUS reporter gene. Expression of native and mutant *Adh* promoter regulated GUS constructs will be assayed using both particle bombardment of *Arabidopsis* seedlings and PEG-$Ca(NO_3)_2$ transformation of *Arabidopsis* protoplasts.

Domains or sequence elements necessary for activation of *Adh* transcription are thereby identified, and a comparison will be made between these results and those obtained in the previous chapter.

Finally, a method for transformation of *Arabidopsis*

seedlings by silicon carbide fiber injection of plasmid DNA will be described, and selected *Adh* constructs will be examined by this method in order to evaluate its usefulness as a tool for gene expression studies.

Materials and Methods

Plant materials

Arabidopsis cells in suspension were initiated from seeds germinated on MS media (Sigma) containing 30% sucrose, vitamins, myo-inositol, 0.8% agar, and 0.5 mg/L 2,4-d. Once established, callus was transferred to liquid media described above. Cell suspensions were maintained at room temperature and passaged weekly. *Arabidopsis* seeds were sterilized according to Valvekens et al, (1989) and germinated in sterile MS plates without 2,4-D, or on potting soil in flats. Protoplasts were prepared according to the method of Hauptmann et al. (1987).

Gel Retardation assay

Conditions for gel retardation assay were identical to those used in the previous chapter. Oligonucleotides corresponding to the -210 probe used in the previous study were synthesized to incorporate two contiguous base pair substitutions at locations specified in the text, and were used as conpetitor DNA for this assay..op

Construction of Expression Vectors

The GUS reporter gene system (Jefferson, 1987) was selected based on the absence of detectable beta-glucuronidase (GUS) in *Arabidopsis*. The GUS expression vector pBI221 (Clonetech), shown in Figure 3-1 (Jefferson et al., 1987) contains the GUS coding sequence and NOS terminator cloned into the polylinker of pUC 19. A CAT expression vector (obtained from L. C. Hannah), also derived from pUC19, was used for the construction of *Adh* 5' chimeric plasmids in addition to pBI221.

Preparation of *Arabidopsis Adh* 5' flanking sequences for cloning into the expression vector was begun by the removal of a 2.2 kb EcoR1 fragment from the *Arabidopsis Adh* subclone, jAT3011 (obtained from E. Meyerowitz). This fragment contained 1.0 kb of 5' flanking sequence in addition to 1.2 kb of *Adh* coding sequence. This fragment was then cloned into the EcoR1 site of M13 for preparation of ssDNA. *In vitro* mutagenesis was performed by the method of Kunkel et al.(1987). Oligonucleotides previously synthesized for gel retardation analysis of G-box mutations were utilized for the incorporation of those base substitutions into the full length 5' flanking

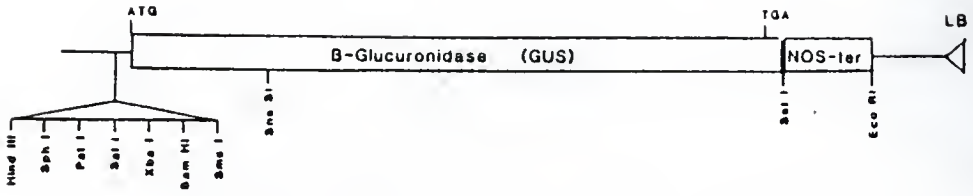


Figure 3-1. GUS expression vector.

This vector was construct by cloning the bacterial glucuronidase (GUS) coding region into the polylinker region of plasmid pUC 19. From Jefferson et al., 1987.

flanking sequence. Wild type or mutant (GM1 or GM2) single stranded DNA from M13 was then used as a template for PCR synthesis of fragments suitable for cloning into the expression vectors (Figure 3-2).

Additional oligonucleotides were synthesized to be used as PCR primers for preparation of *Adh* 5' cloning fragments. The primers (17 to 19 bp long) were designed to anneal either to the bottom strand at +30 relative to transcription start, or the top strand at -940, -855, -652, -482, -390, -289, -177, -177 and -77. Furthermore, these primers were designed to incorporate either a BamH1 site at the 3' fragment end (+30 is 28 bp 5' of *Adh* translation start), or an Sst1 site at the various "deletion" end points (Figure 3-3). Wild type or mutant single stranded template was combined with the appropriate primers for double stranded DNA synthesis and subsequent PCR amplification. PCR products obtained by this method (shown in Figure 3-4) are practically homogeneous and require only a minimum of preparation for the digestion of 5' and 3' ends with restriction enzymes BamH1 and Sst1.

The selection of 5' Sst1 and 3' BamH1 sites described above was based on the CAT expression vector, which contains the BamH1 site closest to the CAT coding region. Additional manipulations were required for cloning into pBI221, and this is described in Appendix B.

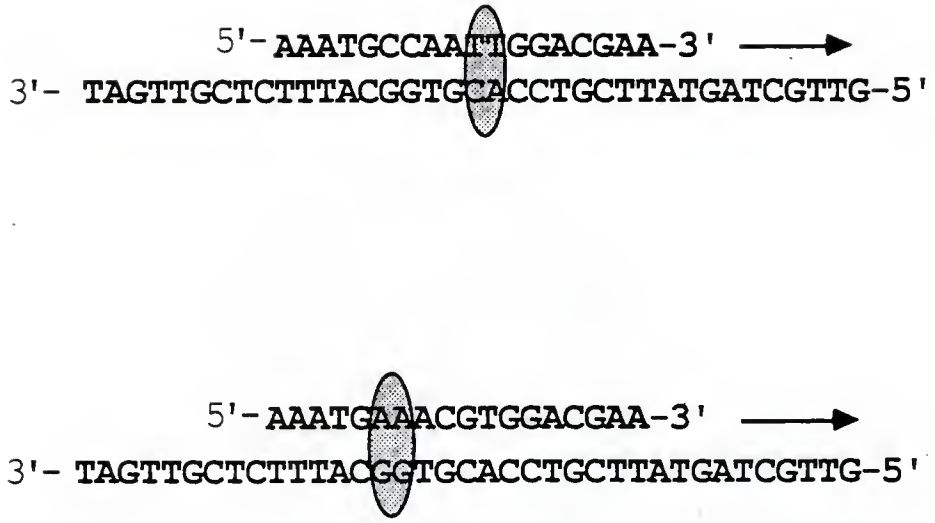


Figure 3-2. Mutagenic oligonucleotide binding to *Adh* -210 region for *in vitro* mutagenesis

Oligonucleotides were designed to incorporate a contiguous two base pair substitution at selected locations of the G-box was used for *in vitro* mutagenesis. Arrows indicate the direction of second strand synthesis.

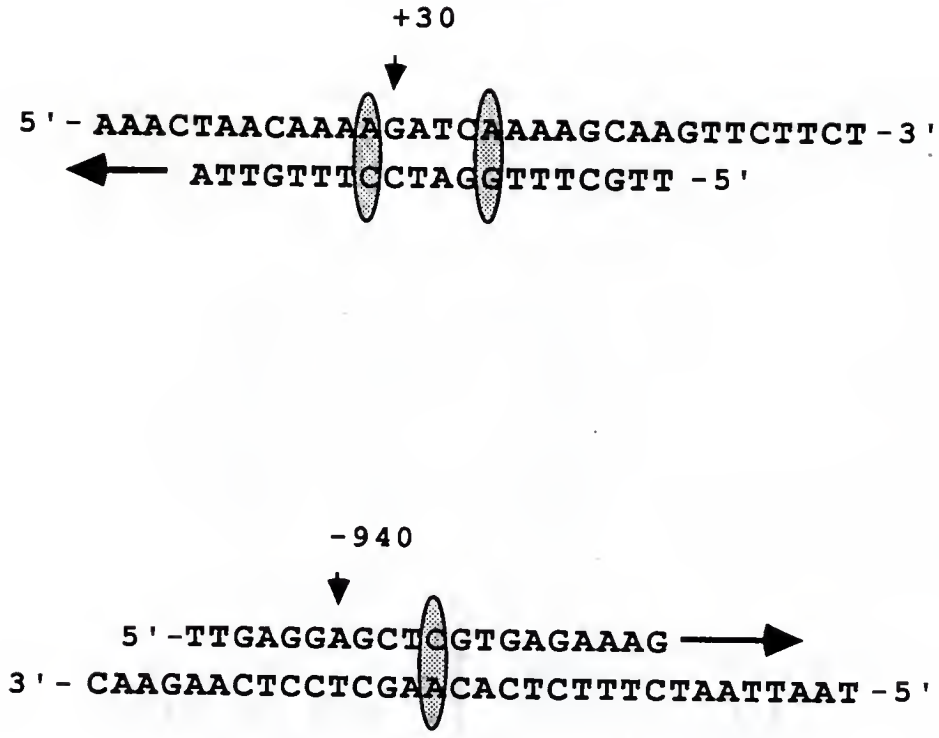


Figure 3-3. PCR priming and mutagenesis for deletion series construction.

The oligonucleotide PCR primers shown above (next to arrows) were designed to anneal to either the *Arabidopsis Adh* gene top strand at +30 (shown above) incorporating a new BamHI site, or the bottom strand at various locations on the 5' flanking sequence and incorporating an SstI site. Arrows indicate direction of DNA synthesis.

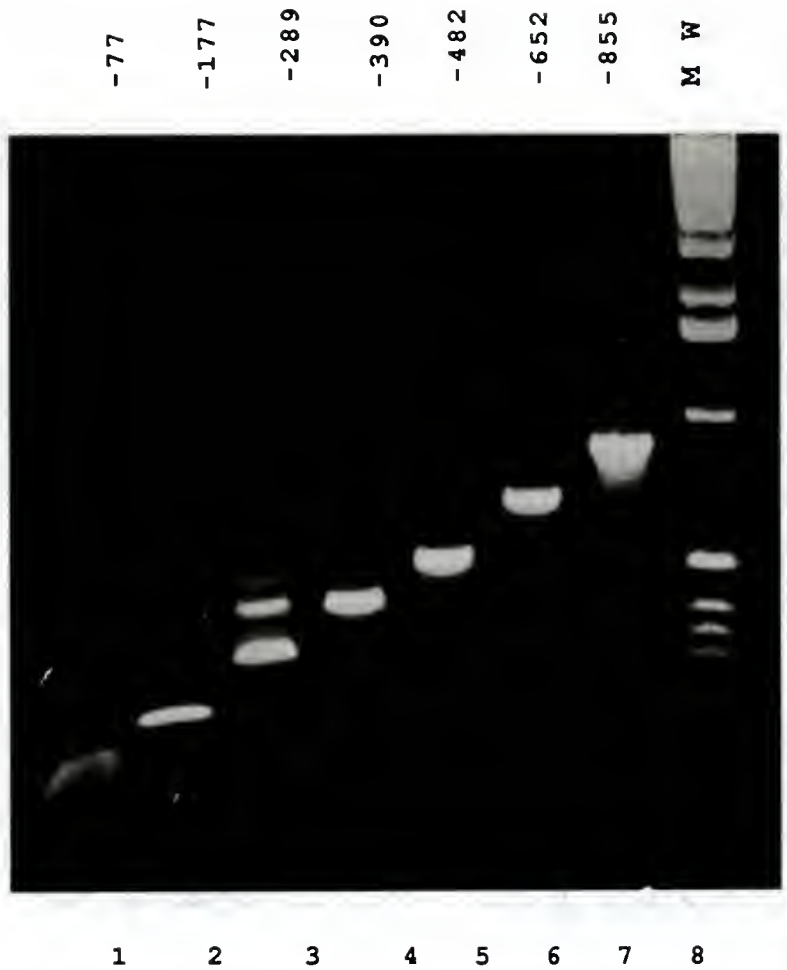


Figure 3-4. PCR synthesis of *Arabidopsis Adh* deletion fragments.

The products of PCR reactions using ssDNA template and primers described in Figure 3-3 are shown above. Fragment sizes are 100 bp (lane 1), 200 bp (lane 2), 330 bp (lane 3), 420 bp (lane 4), 510 bp (lane 5), 690 bp (lane 6), and 880 bp (lane 7). Molecular weight markers are shown in lane 8 (kb ladders).

Particle Bombardment

The method of gene transfer by high velocity microprojectiles (Klein et al., 1988a; Klein et al., 1988b) was used for transformation of 4 day old *Arabidopsis* seedlings. Five ug of supercoiled plasmid DNA was precipitated onto 1.2 um diameter tungsten particles for subsequent bombardment. The bombardment device ("gun") used for these experiments was designed and constructed by this facility. Samples (still in the growth plate) were inserted into the vacuum chamber of the gun (all within a laminar flow hood) and a negative pressure of 26 mm Hg was obtained prior to bombardment. Samples were then removed and plates were wrapped in parafilm. Following a 24 hr incubation at 24^{o} C, 16 hr light/ 8 hr dark, the samples were removed for histochemical staining (Jefferson, 1987) at 37^{o}C for 24 hr.

Transformation by silicon carbide fiber injection

Arabidopsis was grown for 3 days on MS (2,4-D) plates as previously described for particle bombardment. Four day old seedlings are not amenable to this transformation method. On the 3rd day of growth, seedlings are removed from plates and transferred to 1.5 ml eppendorf tubes (approximately 50 seedlings per tube). Transforma-

tion was carried out as described by Kaeppler et al.(1990) with the following modifications (Appel et al., 1988) which are designed to ostensibly precipitate the DNA onto the fiber; the transformation mixture contained 60 ug of supercoiled plasmid DNA, 125 mM $CaCl_2$, and 1.0 mM sodium phosphate, pH=7.0.

Samples are vortexed for one min, transferred to plates, aspirated, and immersed in 400 ul of MS media. Following 24 hr incubation, the samples are removed for staining as previously described.

Protoplast transformation

Arabidopsis protoplasts were harvested in lots of between 4.0 (10^7) and 8.0 (10^7), and allowed to incubate for 30 min in 14 ml of MS/2,4-D media containing 0.4 M mannitol. The protoplasts were then centrifuged for 10 min, 900 rpm, RT. The pellet was resuspended in MaMg (Negrutiu et al., 1987) to a final protoplast concentration of 10^7/ml. Transformation was performed by the method of Damm et al. (1989) using 50 ug each of test and reference plasmid DNA (-950 *Adh*/CAT or 35S/luciferase, obtained from H. Klee), 100 ug of salmon sperm DNA as carrier, and a final concentration of 20 % PEG. Following the 30 min PEG-CMS incubation, the transformation mixture was transferred to 5.0 ml of MS (2,4-D) with 0.4

M mannitol in a 10 cm petri dish and wrapped in parafilm. Samples were incubated 18 hr at RT in darkness. Protoplasts were recovered by centrifugation at 5000 g for 15 min. Following resuspension in 200 ul of the appropriate extraction buffer, protein was extracted by grinding in a 1.5 ml eppendorf tube for 30 sec. Cell debris was removed by a 2.0 min centrifugation at 4^{o}C, and supernatant transferred to a fresh tube for assay of gene activity.

Assay of gene activity

GUS assays, histochemical or flourometric, were performed according to Jefferson (1987). Biochemical measurement of GUS for subsequent fluorimetric assay included approximately 1.0 to 10 ug (100 ul) of total protein (Bradford assay) in a final volume of 500 ul of assay buffer. CAT activity was measured by the method of Gorman (1982). Activity of the firefly luciferase reference gene was measured by a modification of the method of Howell et al. (1989). (Protoplast transformations were assayed separately for GUS and luciferase activity.) The luciferase extraction buffer contained 0.1 M K phosphate buffer, pH=7.8, 2.0 mM EDTA, 2.0 mM DTT, and 5% glycerol. Luciferase assay reactions contained 10 ul of extract (0.1-1.0 ug total protein), and 200 ul of reaction buffer (25 mM tricine, pH=7.8, 15 mM $MgCl_2$, 5 mM ATP, and 0.5 mg/ml BSA.

Results

Gel retardation assay of G-box mutations

The 18 bp G-box oligonucleotide (-210) used for characterization of G-box binding was used to evaluate the influence of base substitutions on GBF binding affinity. Those bases selected for substitution were highly conserved G residues (Giuliano et al., 1988) known to interact with protein from *in vivo* and *in vitro* DMS footprinting (Ferl and Laughner, 1989; results of Chapter 2). The base sequences of native G-box and G-box mutations 1 (GM1) and 2 (GM2) oligonucleotides are shown in Figure 3-5.

Binding affinity assay of GM1 and GM2 is shown in Figure 3-6. Crude whole cell extracts from cells in suspension (lanes 1 to 4) or mature leaves (lanes 5 to 8) are shown to bind the -210 oligonucleotide probe as previously described in Chapter 2 (lanes 1 and 5). Homologous -210 competitor DNA is effective in competing for cell culture (lane 2) or leaf (lane 6) GBF binding at 50 ng. An equivalent amount of mutant G-box competitor does not effectively compete for GBF binding to -210 probe (lanes 3, 4, 7, and 8). Partial competition is observed for GM1 competitor using both cell culture (lane 3) and leaf (lane 7) extract. GM2 competitor, however, is apparently unable to bind GBF and does not compete for GBF binding to -210 probe.

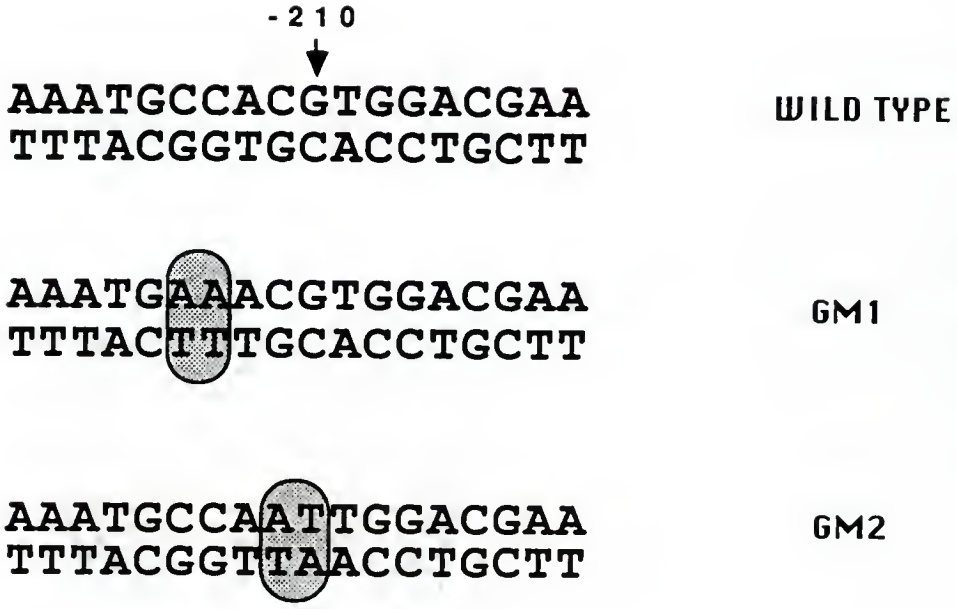


Figure 3-5. Display of the Adh G-box Probe and Adh G-box Mutant Competitor Oligonucleotides.

Specific G-residues of the Adh G-box, which had been shown previously (Figures 2-7, and 2-8) to contact protein, were selected for substitution in the synthesis of mutant competitor oligonucleotides. Two locations of mutations were seleced, distal and central, with reference to the GTGG dyad axis of symmetry (shown above, GM1 and GM2 respectively). Contiguous two base substitutions were selected with the intention of complete disruption of protein binding.

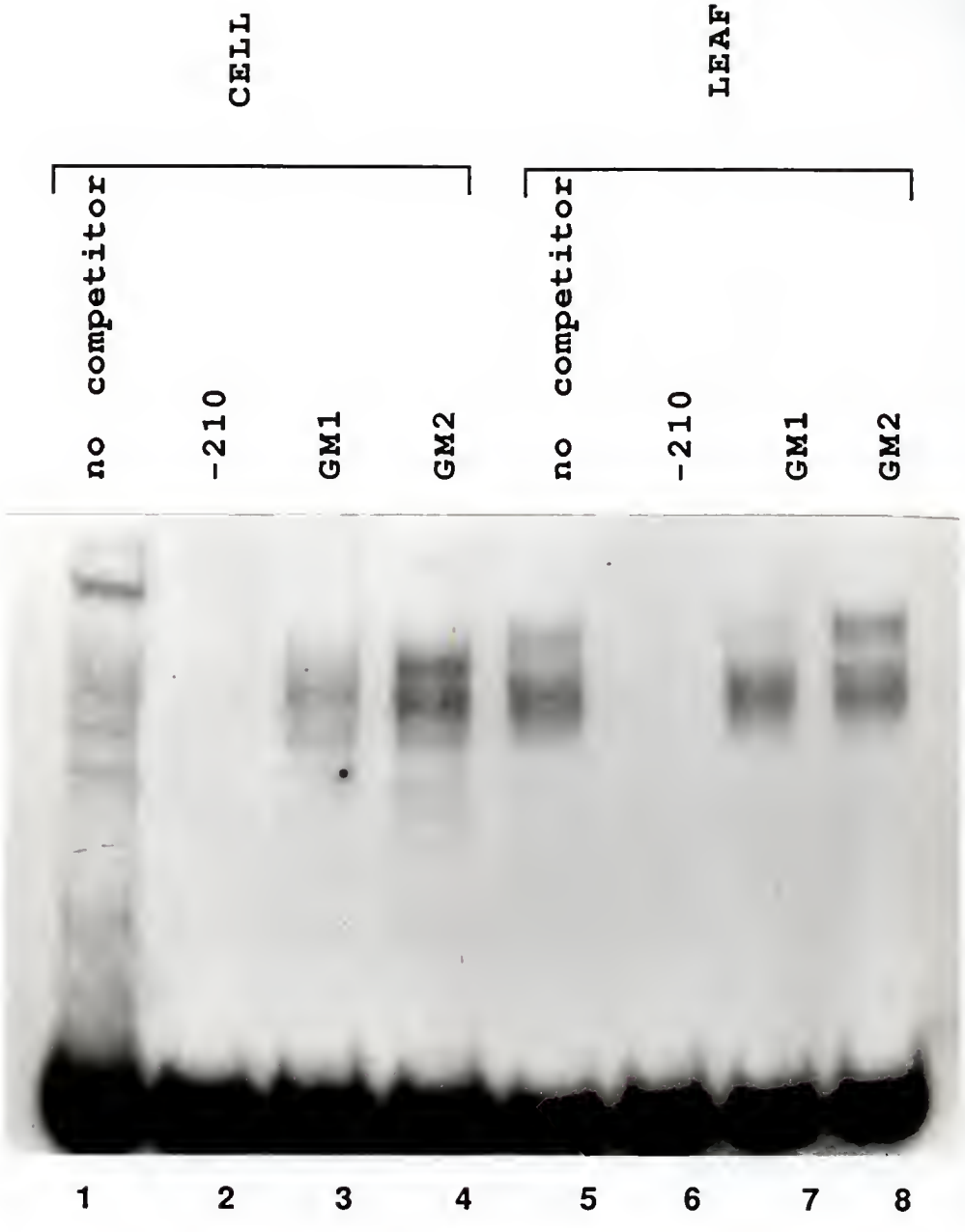


Figure 3-6. Mutation of The Adh G-box disrupts protein-DNA binding.

The Adh G-box probe is bound by a protein component of Arabidopsis crude whole cell extract from cell cultures (lane 1) and mature leaves (lane 5) in the absence of poly(dIdC). G-box/protein binding is not observed in reactions which contain cold Adh G-box competitor DNA (lanes 2 and 6). The mutant oligonucleotide competitor DNAs, GM1 (lanes 3 and 7) and GM2 (lanes 4 and 8), do not compete for Adh G-box protein binding.

Transformation of *Arabidopsis* Seedlings by Particle Bombardment

The 1.0 kb 5' flanking region of *Arabidopsis Adh*, and deletions or mutations thereof, were cloned into the GUS expression vector pBI221 (Figure 3-7). These chimeric constructs were used for the analysis of *Adh* regulated transient expression in transformed seedlings and protoplasts.

Intact, four day old *Arabidopsis* seedlings were subjected to transformation by particle bombardment. No GUS activity was observed in seedlings which were bombarded with either no DNA or the CAT expression vector (Figure 3-8,A). Seedlings bombarded with -940/GUS contain numerous spots in all tissues (Figure 3-8, B), as do those which were bombarded with -855/GUS, -652/GUS, -482/GUS, -390/GUS, and -289/GUS (Figure 3-8; C, D, E, F, and G). Each of these samples possess roughly equivalent levels of GUS activity as estimated by the number of spots and the intensity of blue color. These samples also appear to have spots approximately equally distributed among tissues, with the exception of -390/GUS which has an elevated number of spots in root tissue.

In contrast, very few blue spots are observed for -177/GUS (Figure 3-8, H). GUS expression was not detected in the -77/GUS (Figure 3-8, I) construct.

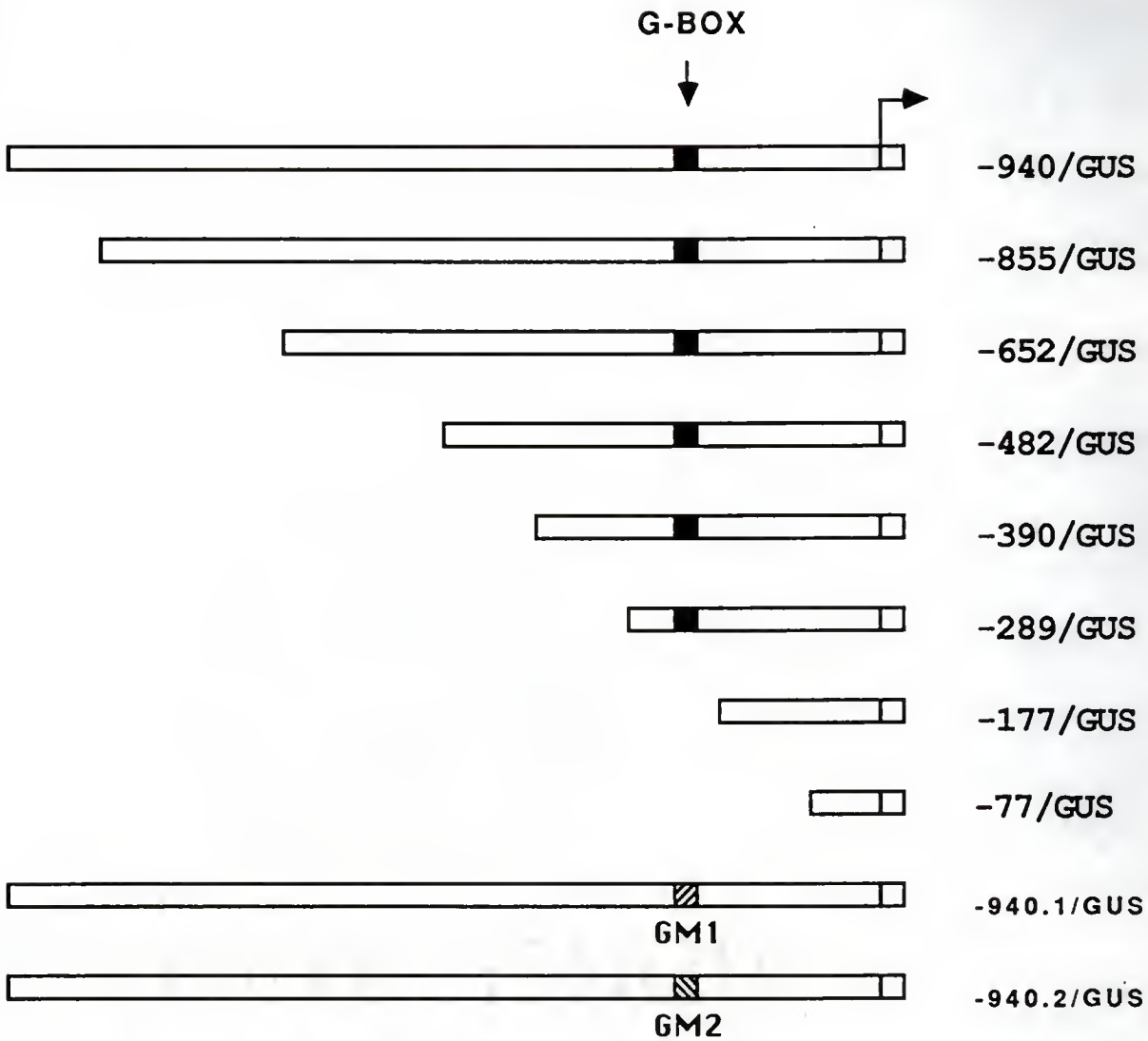


Figure 3-7. Arabidopsis Adh 5' Deletion constructs and G-box mutations.

The 5' flanking sequence of Arabidopsis Adh was cloned into the GUS expression vector pBI221 (-940/GUS). Deletions from the 5' end of the Adh flanking sequence were prepared at approximately 100 bp intervals from -940, and these were also cloned into pBI221 (-855/GUS, -652/GUS, -482/GUS, -390/GUS, -289/GUS, -177/GUS, and -77/GUS).

The Adh G-box mutations described in Figure 3-1 (GM1 and GM2) were also incorporated into the full length promoter via. site directed mutagenesis, and these fragments were cloned into pBI221 as well

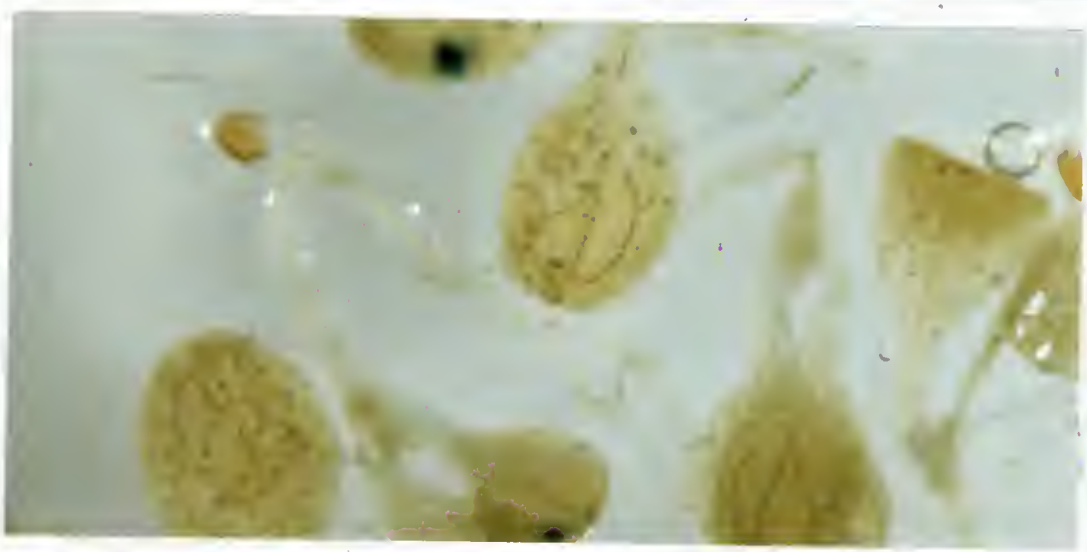
These Arabidopsis chimeric constructs were used for all transient expression experiments to follow.

Figure 3-8. Transformation of intact seedlings by particle by Particle Bombardment.

Four day old seedlings were transformed with the full length promoter, -940/GUS (A) by microprojectile bombardment. Samples contained roughly 50 seedlings each, and each construct was transformed in duplicate. Results from promoter deletion constructs are shown in B (-855/GUS), C (-652/GUS), D (-482/GUS), E (-390/GUS), F (-289/GUS), G (-177/GUS), and H (-77/GUS).

A

B



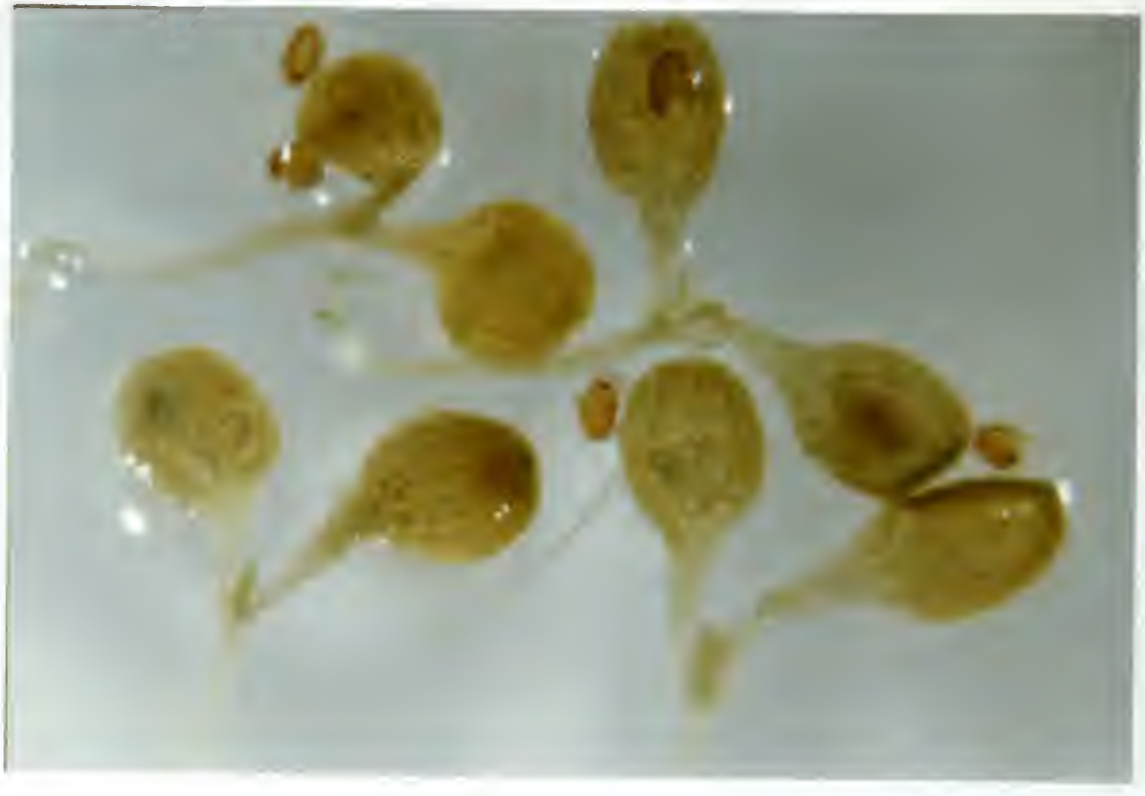
C

D)

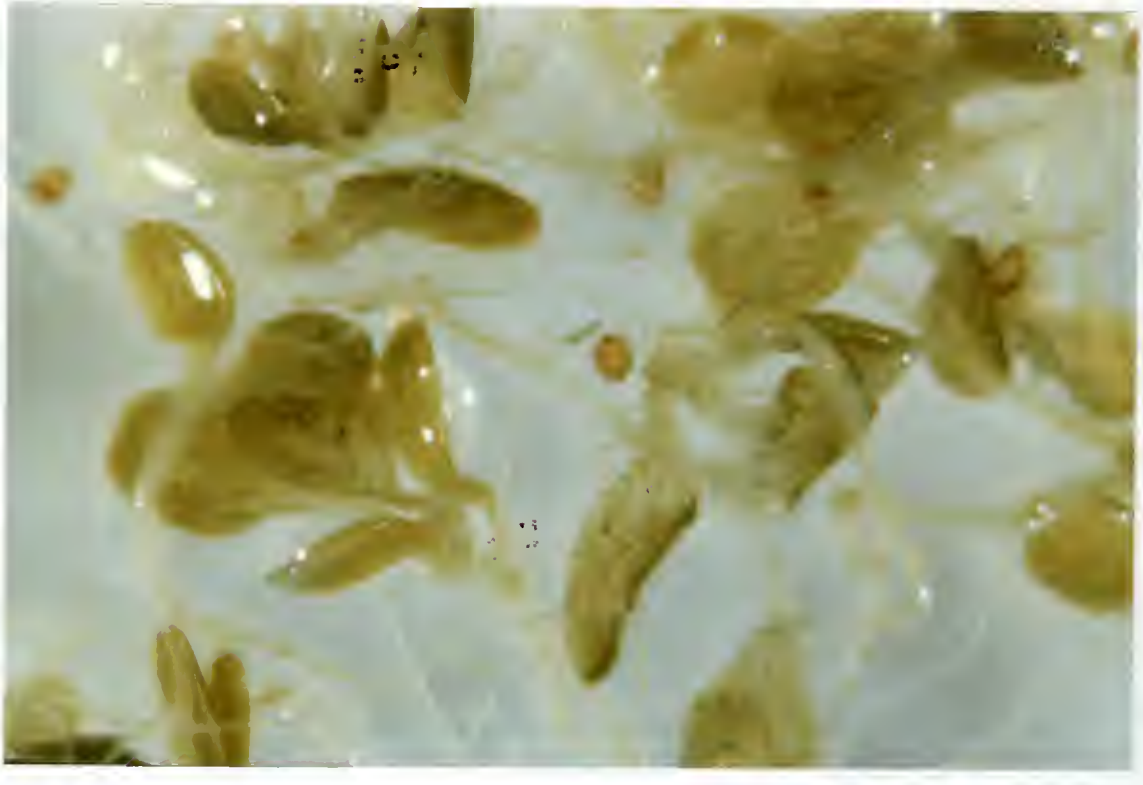
E

F

G



H



Each construct photograph is the combination of two replicate samples from a single experiment, and replicates were generally in close agreement.

Particle bombardment of 4 day old seedlings was also conducted using the -940/GM1/GUS and -940/GM2/GUS constructs (Figure 3-9, A and B). GUS activity is observed for both mutant constructs, with the number and intensity of blue spots being only slightly reduced. A summary of these results is provided in Table 3-1.

Mature Arabidopsis seedlings were also examined by this method for Adh regulated expression (Figure 3-10). Fourteen day old seedlings were transformed with -940/GUS and subsequently allowed to incubate with or without oxygen. GUS activity is observed in leaf tissue only, and did not significantly vary with oxygen levels. The relative absence of root activity (one spot in the -0_2 sample) most likely reflects the low frequency of transformation inherent in this method. Expression of GUS in 4 day old seedlings was not significantly altered by oxygen depletion (data not shown).

Particle bombardment was attempted for cells in suspension using a variety of cell densities and cell ages. GUS activity was not detected in these experiments using either 35S/GUS or -940/GUS.

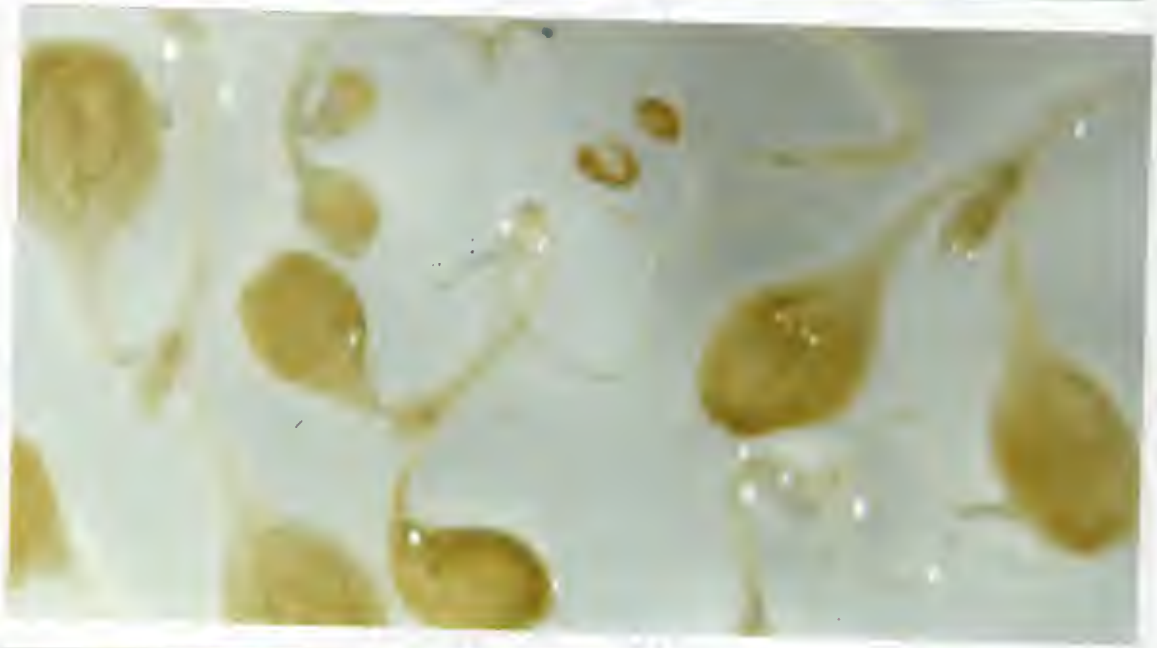
Biochemical measurement of GUS activities in seedlings was prevented by high background fluorescence.

Figure 3-9. Transformation of *Arabidopsis* seedlings with -940.1/GUS and -940.2/GUS.

Seedlings were transformed as in Figure 3-8 but with either -940/GUS (A), or the mutant constructs -940.1/GUS (B) and -940.2/GUS (C).

A

B



C

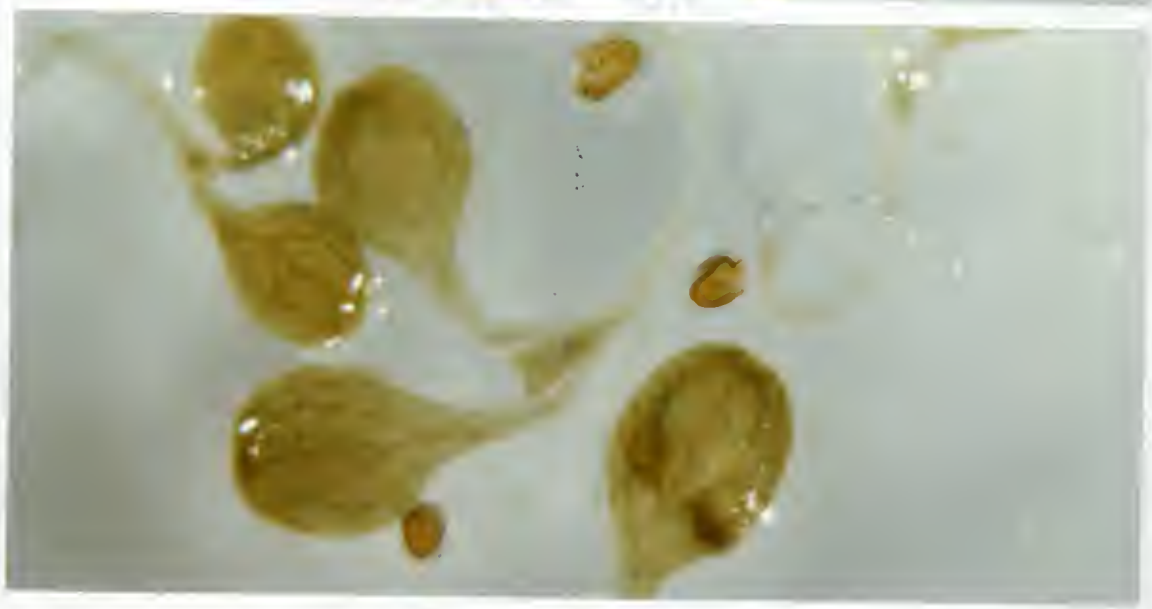


Table 3.1

Summary of Particle bombardment transformation results

Construct	Activity
-940/GUS	+++
-855/GUS	++++
-652/GUS	+++
-482/GUS	++
-390/GUS	+++
-289/GUS	+++
-177/GUS	+
-77/GUS	-
-940.1/GUS	++
-940.2/GUS	++

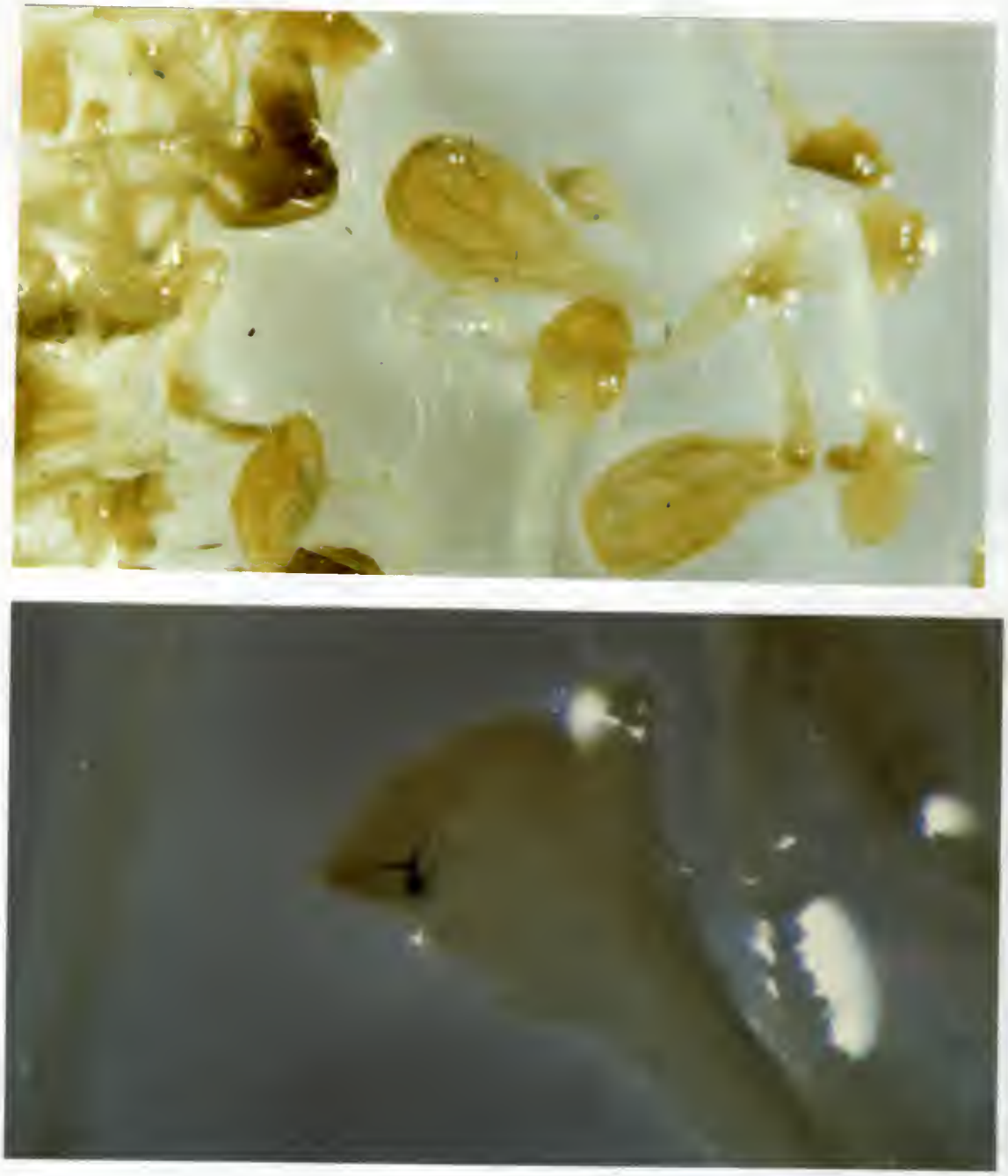


Figure 3-10. Transformation of mature *Arabidopsis* Seedlings.

Thirteen day old seedlings were transformed by particle bombardment with -940/GUS. Fewer seedlings were transformed and assayed due to seedling size. Root tissue transformation was not frequent.

Transformation by Silicon Carbide Fiber "whiskers" Injection

Silicon carbide fiber transformation was attempted using seedlings at 1, 2, 3, and 4 days after germination. Successful transformation was observed for 3 day old seedlings only. In general, this method was far less efficient than particle bombardment for transformation. Transformed samples contained 1 to 5 spots, and spot intensity varied considerably among replicates. When the full length promoter construct (-940/GUS) was compared with -289/GUS, and -177/GUS, activity was observed in only the -940/GUS and -289/GUS treatments (Table 3-2).

An occasional blue staining was observed in the shoot apex which appeared to have either spread extensively from the point of origin, or was a result of expression in more than one cell. This was observed in three seedlings, and in each case the shoot apex or leaf primordia were uniformly stained a dark blue or indigo. One of those seedlings is shown in Figure 3-11. It should be noted here that Arabidopsis seedlings are growing rapidly by the third day after germination, and continue to grow for the 24 hr following bombardment, prior to staining.

Seedlings which had been transformed were examined (G. Erdos) for fiber injection with scanning electron

Table 3-2

Summary of Whiskers Transformation

Construct	Activity
-940/GUS	+++
-855/GUS	+++
-390/GUS	+++
-289/GUS	+++
-177/GUS	-
-77/GUS	-

Figure 3-11. Transformation of *Arabidopsis* by "whisker" injection.

Three day old seedlings were transformed by the "whiskers" method using -940/GUS. Seedlings remain intact following one minute vortexing at high speed. Blue stain appears in leaf primordia.

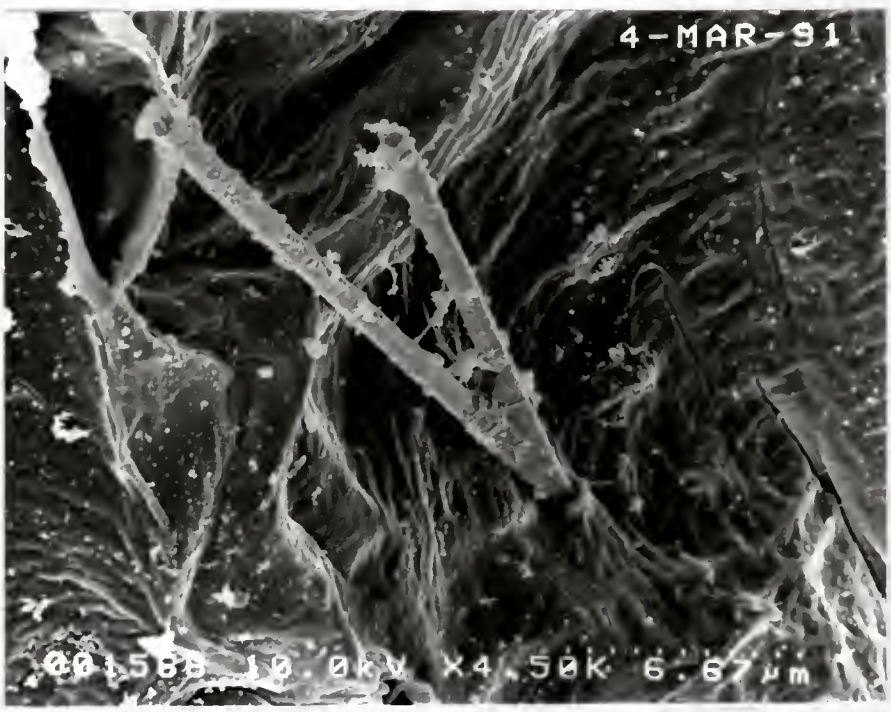


Figure 3-12. Electron Microscopy of "whisker" injection.

Seedlings subjected to transformation by silicon carbide fiber injection were fixed and prepared for SEM. This fiber is atypical in that it is bifurcated at one end. Many fibers could be found in contact with the plant surface without actual penetration.

microscopy (Figure 3-12). Many fibers were found in contact with the plant surface, but very few had obviously penetrated the cell wall. Penetration was more frequent in leaf tissue than roots.

Transformation of Arabidopsis protoplasts

Protoplasts were prepared on the sixth day of a seven day passaging cycle. Protoplast yield from approximately 5.0 grams fresh weight of suspension culture cells varied between 5 x 10^7 and 10^8. A minimum of 10^6 protoplasts/300 ul was necessary for efficient transformation. Lower protoplast amounts resulted in non-linearity of GUS activity. Cells in suspension appear yellow in color when properly maintained, and unhealthy suspension cultures (brown or pale yellow) generally yielded few protoplasts regardless of the amount of starting material.

Relative GUS activities of the full length, deletion, and mutation constructs (Figure 3-7), using -940/CAT as the reference construct, were examined on 6/21/91 by transformation of protoplasts, and the results are shown in Table 3-3. Following cell harvest and extraction, total protein was measured from 10 ul of extract (column 1) and GUS assays were then performed by measuring the production of 4-methylumbelliferone (4MU) at various time points (columns 2 through 5). The re-

Table 3-3
Total Protein and Fluorescence for Experiment 6/21/91

	Total Protein (ug/ul)	Fluorescence, (nM 4MU) 20'	40'	60'	80'
1	0.563	0.343	0.325	0.349	0.223
2	0.589	1.650	3.073	4.336	5.889
3	0.592	0.350	0.309	0.294	0.228
4	0.601	9.023	18.76	28.76	38.44
5	0.518	3.487	6.820	10.19	12.90
6	0.484	4.775	9.040	13.12	
7	0.398	7.921	15.39	33.89	33.26
8	0.495	2.330	4.430	6.320	8.539
9	0.402	2.220	5.030	6.360	8.624
10	0.452	7.830	15.46	24.70	31.06
11	0.459	3.140	5.968	8.199	11.51
12	0.472	0.520	0.709	0.831	1.048
13	0.474	0.437	0.535	0.539	0.340

Table 3-4

Protein per assay and Fluorescence per mg protein for Experiment 6/21/91

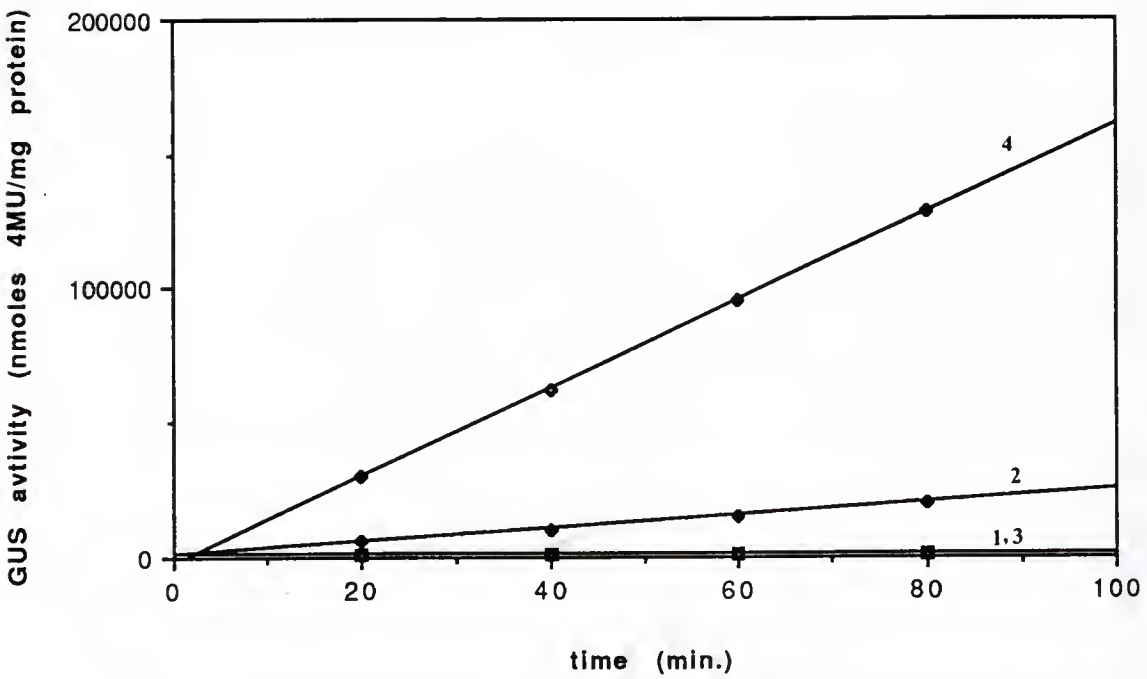
	protein per assay (mg x 10^4)	Corrected Fluoresce (nmoles 4MU/mg protein) 20'	40'	60'	80'
1	2.81	1220	1160	1200	793
2	2.94	5600	10440	14760	20032
3	2.96	1180	1040	1020	770
4	3.00	30060	62520	95880	128160
5	2.59	13460	26320	39300	49841
6	2.42	19720	37320	54180	
7	1.99	39800	77320	112980	167120
8	2.48	9380	17840	25440	34431
9	2.00	11100	25160	31800	43120
10	2.26	34640	68440	109320	137440
11	2.30	13640	25960	35640	50000
12	2.36	2200	3000	3540	4440
13	2.37	1620	2240	2280	1434

sults of Table 3-3 are normalized for protein amounts (Table 3-4) and thenplotted as GUS activity (nM 4MU/mg total protein) as a function of time (Figure 3-13, 3-14, and 3-15).

The linearity of increasing 4MU fluorescence over time is observed with all constructs except the no DNA and 35S/CAT controls, and the -77/GUS construct. Correlation coefficients range from 0.983 for -482/GUS to 1.0 for -940/GUS verifying enzyme activity to be in the linear range. (An increase in activity of the -940/GUS construct is occasionally observed by the addition of the 35S/CAT reference construct). The 35S/CAT was present in all other deletion or mutation transformations.

The slopes of lines plotted in Figures 3-13, 3-14, and 3-15 are then presented as GUS activities relative to the full length construct (Figures 3-16 and 3-17). Activities of the deletion constructs fell into three general categories (Figure 3-16). Deletions to -855 or -390 result in no significant reduction in GUS activity. The -652, -482, and -289 deletion constructs produce less than half of wild type activity, and further deletions to -177 and to -77 essentially abolish gene activity.

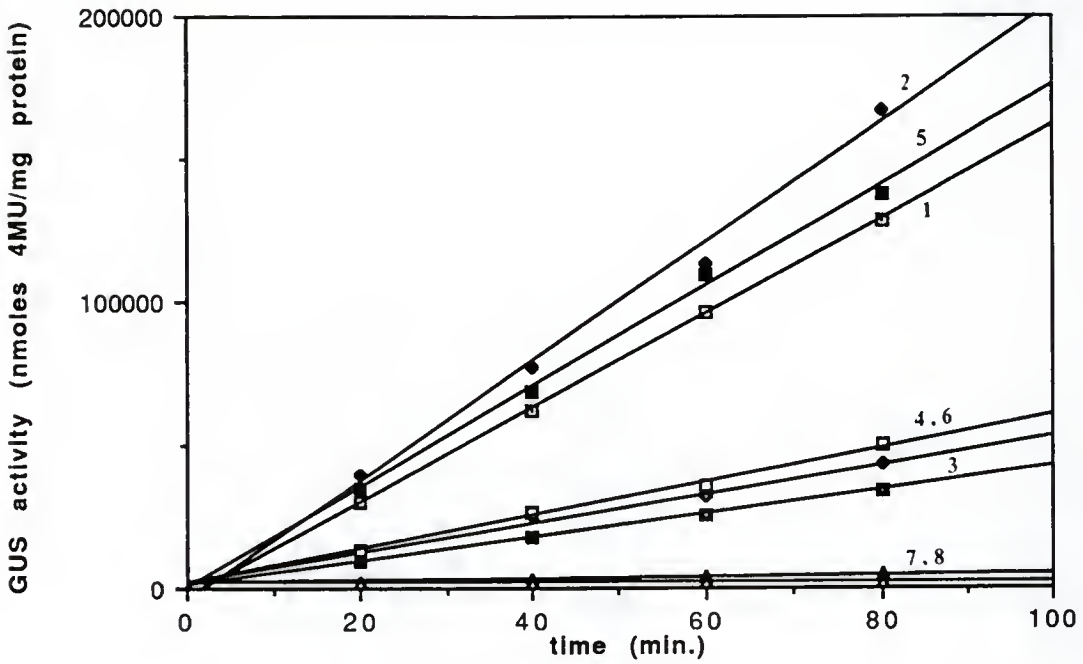
The G-box mutation constructs 940.1/GUS and 940.2/GUS (Figure 3-17) were compared to -940/GUS, and were found to significantly reduce the ability of the full length promoter to activate GUS expression.



1 ◘	no DNA	y = 1403.5 - 6.2050x R^2 = 0.631
2 ◆	-940/GUS*	y = 804.00 + 238.08x R^2 = 0.999
3 ■	35S CAT	y = 1315.0 - 6.2500x R^2 = 0.895
4 ◆	-940/GUS	y = - 2760.0 + 1638.3x R^2 = 1.000

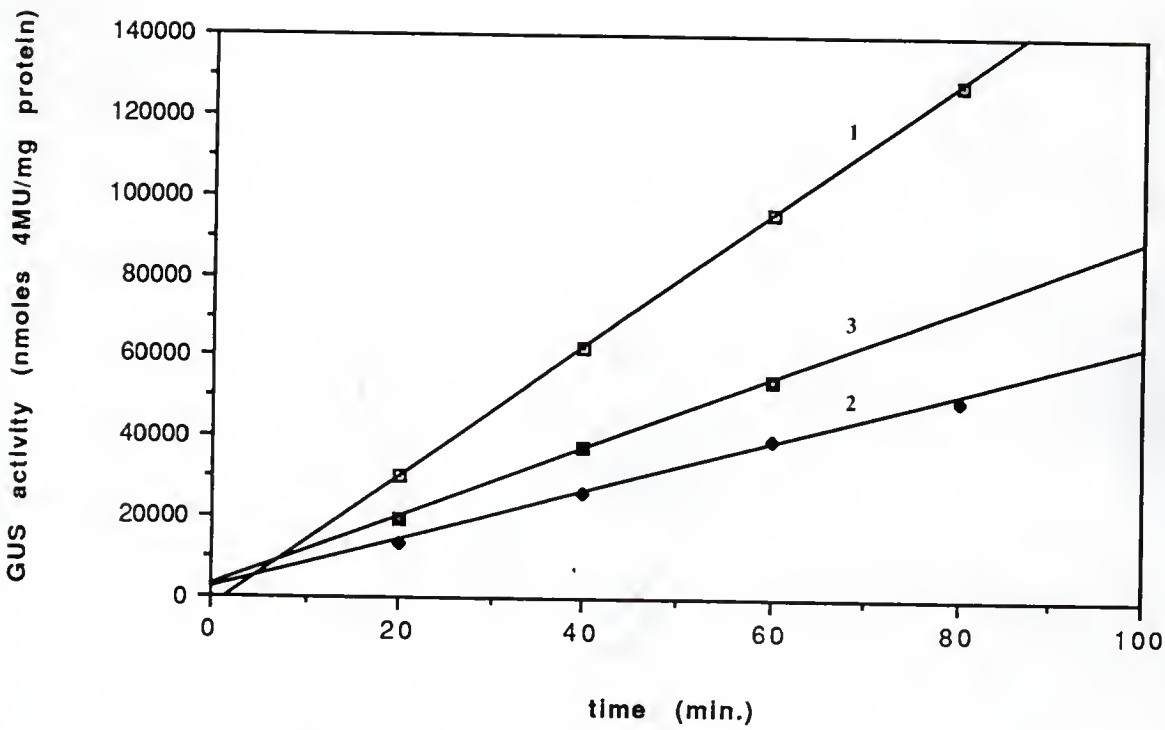
Figure 3-13. Time course of 4MU Fluorescence from experiment 6/21/91.

Fluorescence values used here are from Table 3-4. The slope of each line preceeds the x in each equation. R^2 is correlation coefficient. The -940/GUS * treatment does not include the 35S/CAT reference construct. All other treatments include 35s/CAT.



1 ▫	-940/GUS	y = - 2760.0 + 1638.3x	R^2 = 1.000
2 ◆	-855/GUS	y = - 5100.0 + 2088.1x	R^2 = 0.990
3 ■	-652/GUS	y = 1084.5 + 413.76x	R^2 = 0.999
4 ◇	-482/GUS	y = 2120.0 + 513.50x	R^2 = 0.983
5 ■	-390/GUS	y = 140.00 + 1746.4x	R^2 = 0.995
6 ▫	-289/GUS	y = 1620.0 + 593.80x	R^2 = 0.995
7 ▲	-177/GUS	y = 1480.0 + 36.300x	R^2 = 0.992
8 ▲	-77/GUS	y = 2023.0 - 2.5900x	R^2 = 0.024

Figure 3-14. Time course of Fluorescence of Deletion Constructs from experiment 6/21/91.



1	-940/GUS	y = - 2760.0 + 1638.3x	R^2 = 1.000
2	-940.1GUS	y = 1699.5 + 610.61x	R^2 = 0.998
3	-940.2/GUS	y = 2613.3 + 861.50x	R^2 = 1.000

Figure 3-15. Time course of Fluorescence of G-box mutation constructs from experiment 6/21/91.

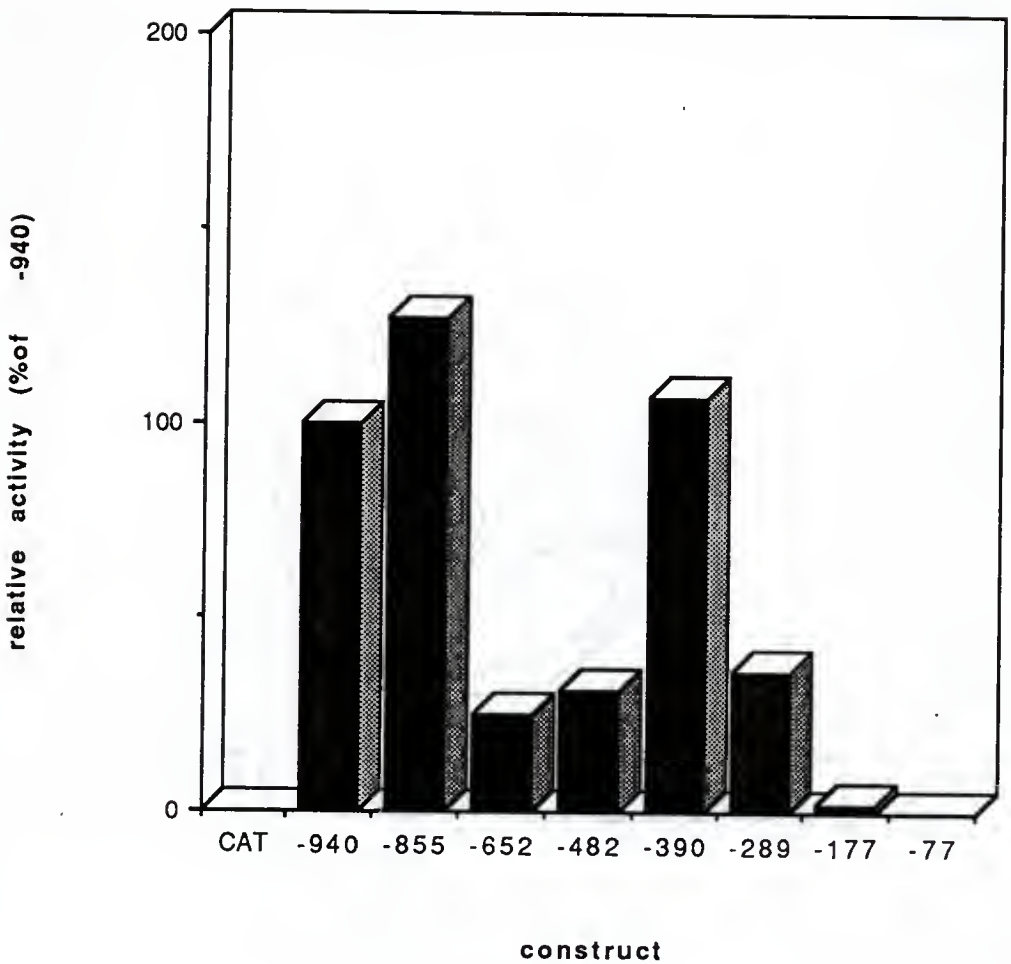


Figure 3-16. GUS activity of Deletion constructs relative to the full length promoter construct,-940/GUS.

Absolute GUS activity for -940/GUS varied between 1.5 and 3.0 umoles 4MU/min/mg total protein among experiments. Relative activities are calculated as a percentage of -940/GUS activity in the same experiment.

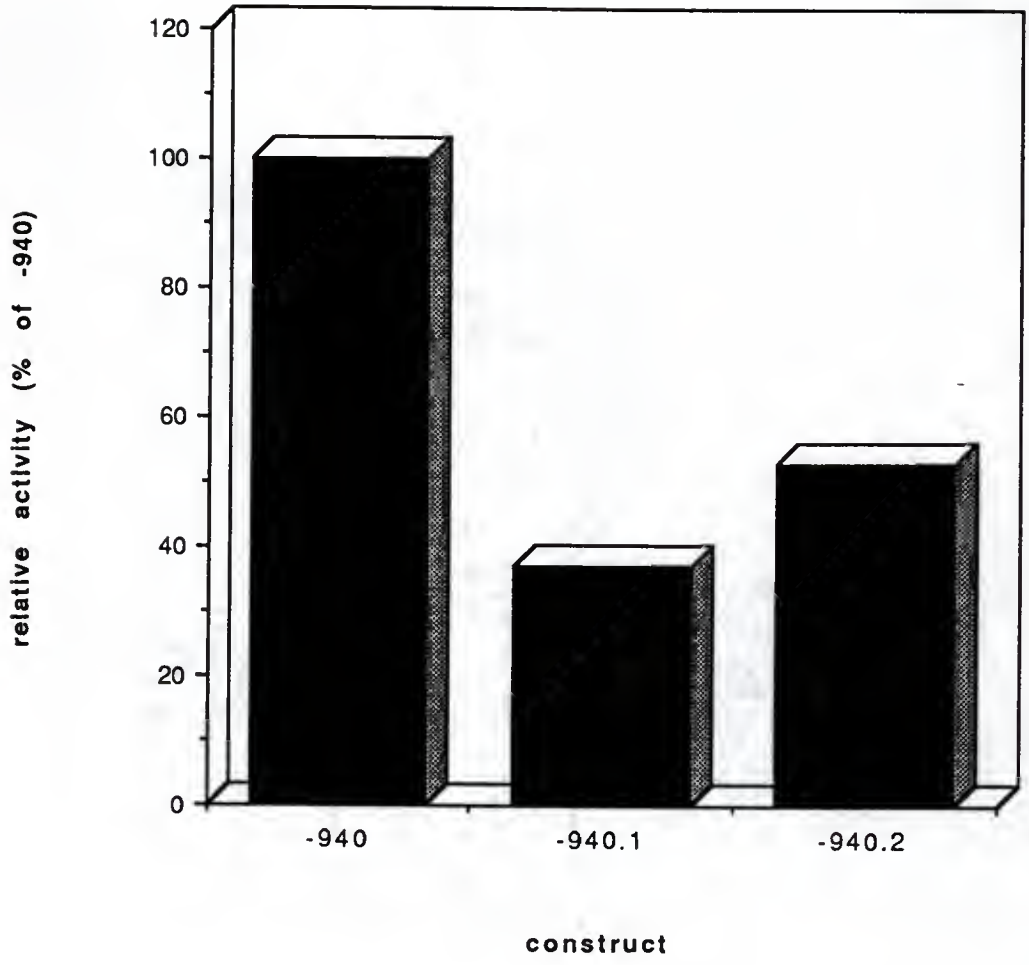


Figure 3-17. GUS activity of G-box mutant constructs relative to the full length promoter construct.

Repeated attempts to obtain measurable CAT activity uaing either 35S/CAT or -940/CAT were unsuccessful. The -940/CAT construct was maintained in the transformation mixture over the following 2 experiments in order to 1) allow for comparisons between this and subsequent experiments, and 2) to function as carrier DNA. This procedure was continued until the procurement of the 35S/Luciferase (Ow et al., 1986) construct.

A technical problem arises when attempting to measure relative activities of several constructs (limiting protoplast yield), as well as time constraints at particular stages of transformation and assay. Initial experiments were designed based on the assumption that activities (relative to full length) should agree from one days experiment to the next. Each construct was transformed (without replication), and the relative activities were analyzed in combination with corresponding activities obtained in subsequent experiments to derive an average relative activity.

Therefore, the previous experiment was repeated on 6/27/91, as shown in Tables 3-5 and 3-6, and these results are plotted in Figures 3-18, 3-19. The resulting GUS activities are displayed in Figures 3-20 and 3-21. A comparison of the results of experiments 6/21/91 and 6/27/91 is presented in Figure 3-22. As opposed to the first experiment, relative activities of the deletion

Table 3-5

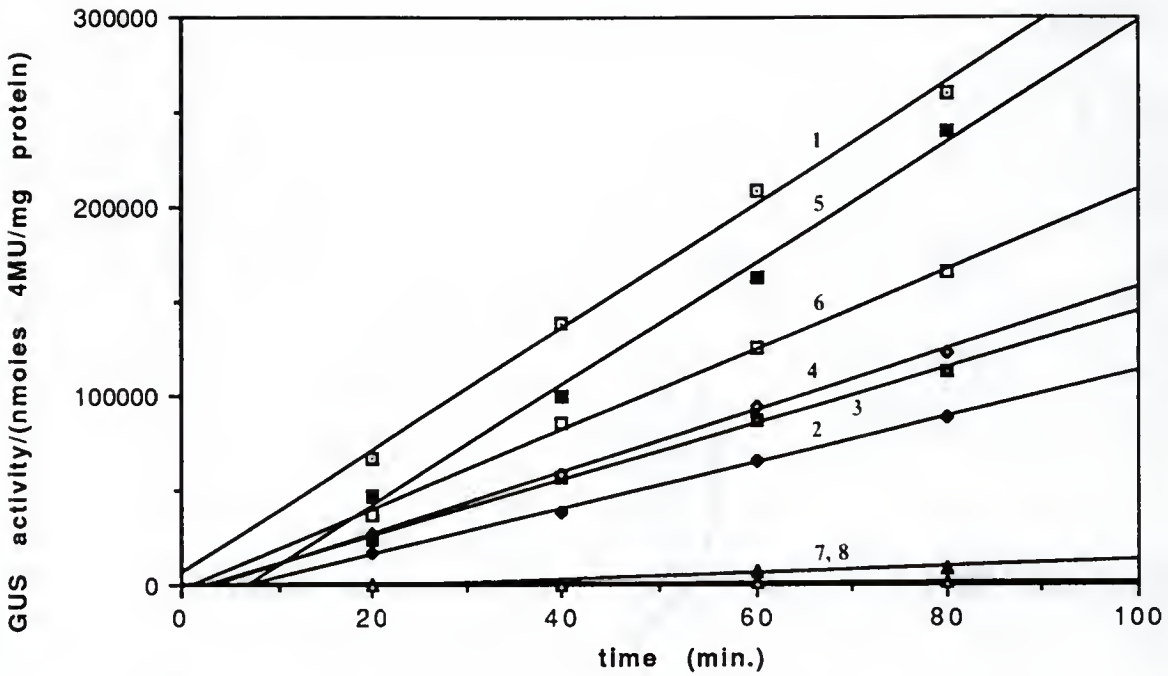
Total Protein and Fluorescence for Experiment 6/27/91

	total protein (ug/ul)	Fluorescence (nM 4MU) 20'	40	60	80
1	0.334	0.000	0.000	0.000	0.016
2	0.411	0.936	1.990	2.967	3.764
3	0.369	1.667	3.456	5.197	6.204
4	0.302	0.000	0.084	0.191	0.396
5	0.328	0.081	0.331	0.537	0.824
6	0.270	0.425	0.977	1.638	2.169
7	0.409	0.610	1.421	2.172	2.765
8	0.399	0.670	1.477	2.369	3.007
9	0.249	1.164	2.515	4.088	5.701
10	0.374	0.926	2.153	2.897	3.976
11	0.332	0.000	0.000	0.178	0.219
12	0.386	0.000	0.000	0.022	0.024

Table 3-6

Protein per Assay and Fluorescence per mg Protein for Experiment 6/27/91

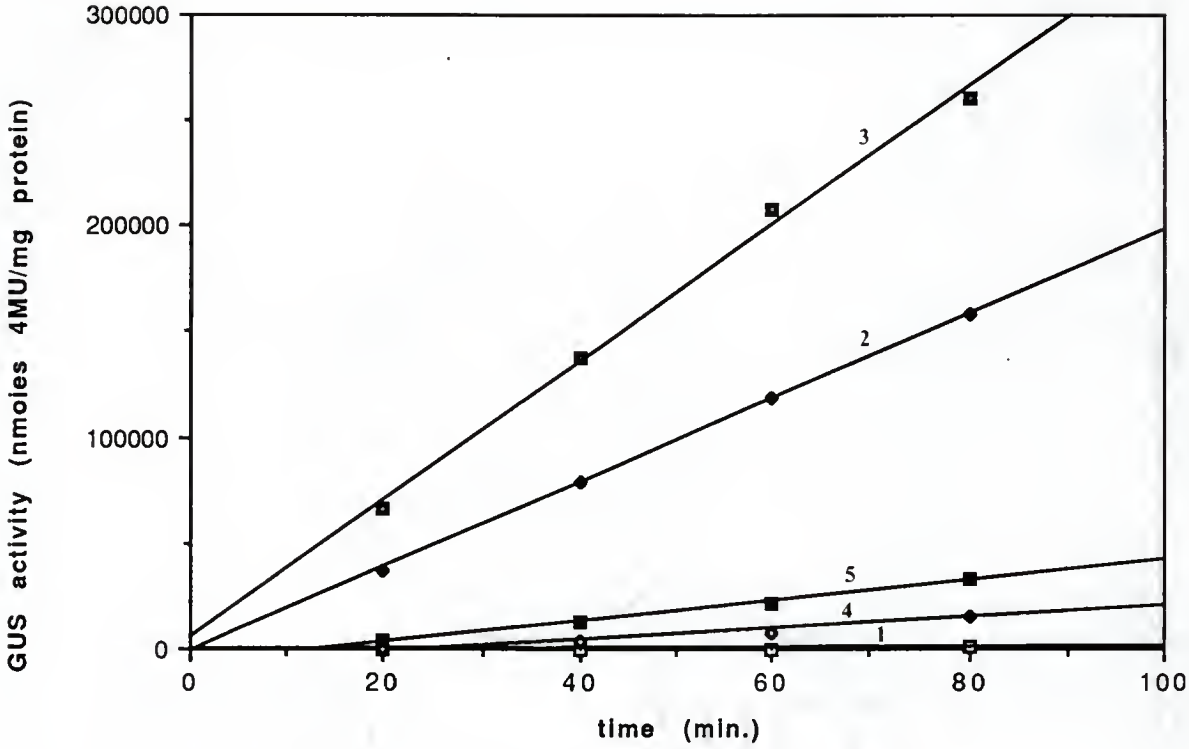
	protein per assay ($mg \times 10^5$)	Corrected Fluorescence (nmoles 4MU/mg protein) 20'	40'	60'80'	
1	0.25	0000	0000	00006440	
2	0.25	37440	79600	118680	158088
3	0.25	66680	138240	207880	260568
4	0.25	0000	3360	7640	15840
5	0.25	3240	13240	21480	32960
6	0.25	17000	39080	65520	88929
7	0.25	24400	56840	86880	113365
8	0.25	26800	59080	94760	123287
9	0.25	46560	100600	163520	239442
10	0.25	37040	86120	125454	165401
11	0.25	0000	0000	7120	8760
12	0.25	0000	0000	880	960



1	-940/GUS	y = 5516.0 + 3256.5x	R^2 = 0.995
2	-855/GUS	y = - 7924.5 + 1211.1x	R^2 = 0.999
3	-652/GUS	y = - 3862.5 + 1484.7x	R^2 = 0.998
4	-482/GUS	y = - 5303.5 + 1625.7x	R^2 = 0.998
5	-390/GUS	y = - 2.2861e+4 + 3207.8x	R^2 = 0.994
6	-289/GUS	y = - 2600.5 + 2122.1x	R^2 = 0.997
7	-177/GUS	y = - 4380.0 + 167.00x	R^2 = 0.866
8	-77/GUS	y = - 480.00 + 18.800x	R^2 = 0.832

Figure 3-18. Time course of Fluorescence for Deletion Constructs from Experiment 6/27/91.

Results are presented here as in Figures 3-13 to 3-15. Slopes of these lines are used to determine absolute GUS activity.



1	no DNA	y = - 320.00 + 9.6000x	R^2 = 0.600
2	-940/GUS*	y = - 1804.0 + 2005.1x	R^2 = 1.000
3	-940/GUS	y = 5516.0 + 3256.5x	R^2 = 0.995
4	-940.1/GUS	y = - 6240.0 + 259.00x	R^2 = 0.955
5	-940.2/GUS	y = - 6620.0 + 487.00x	R^2 = 0.996

Figure 3-19. Time course of Fluorescence of G-box mutation constructs for Experiment 6/27/91.

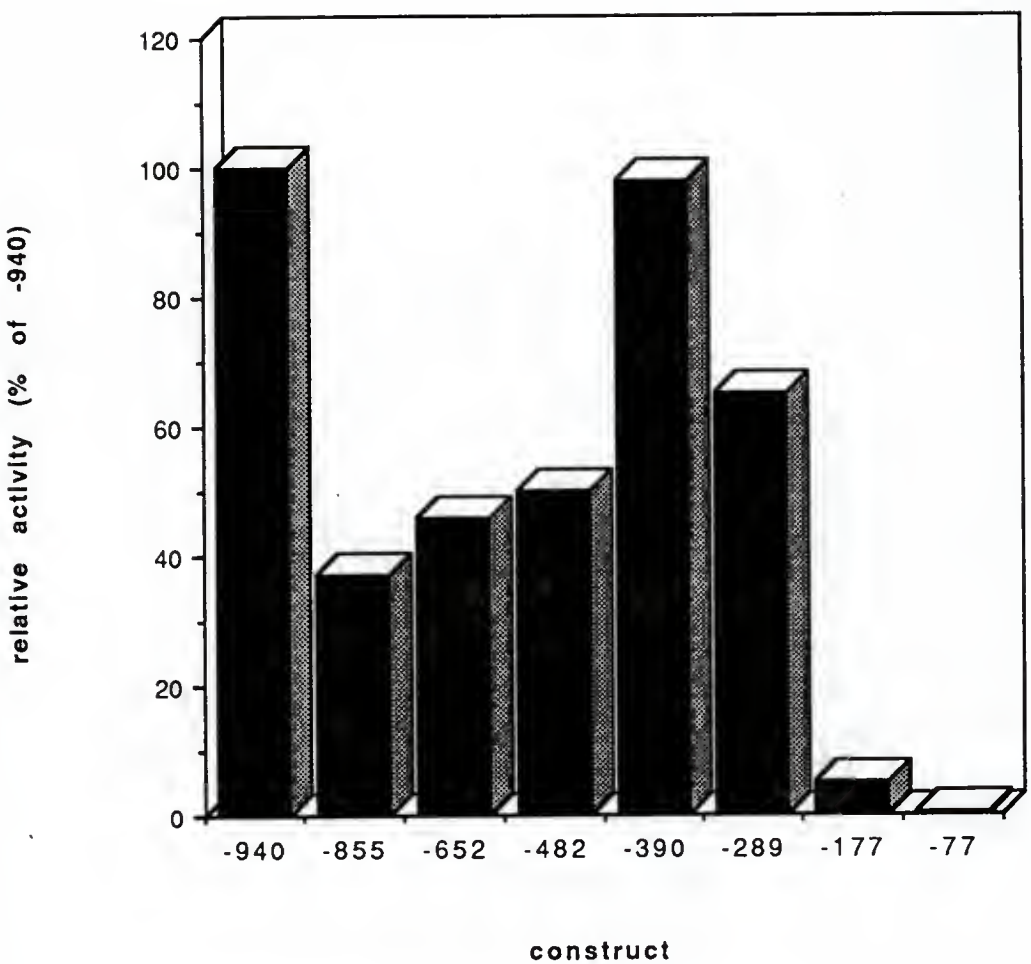


Figure 3-20. GUS activity of Deletion Constructs relative to the Full Length Promoter Construct.

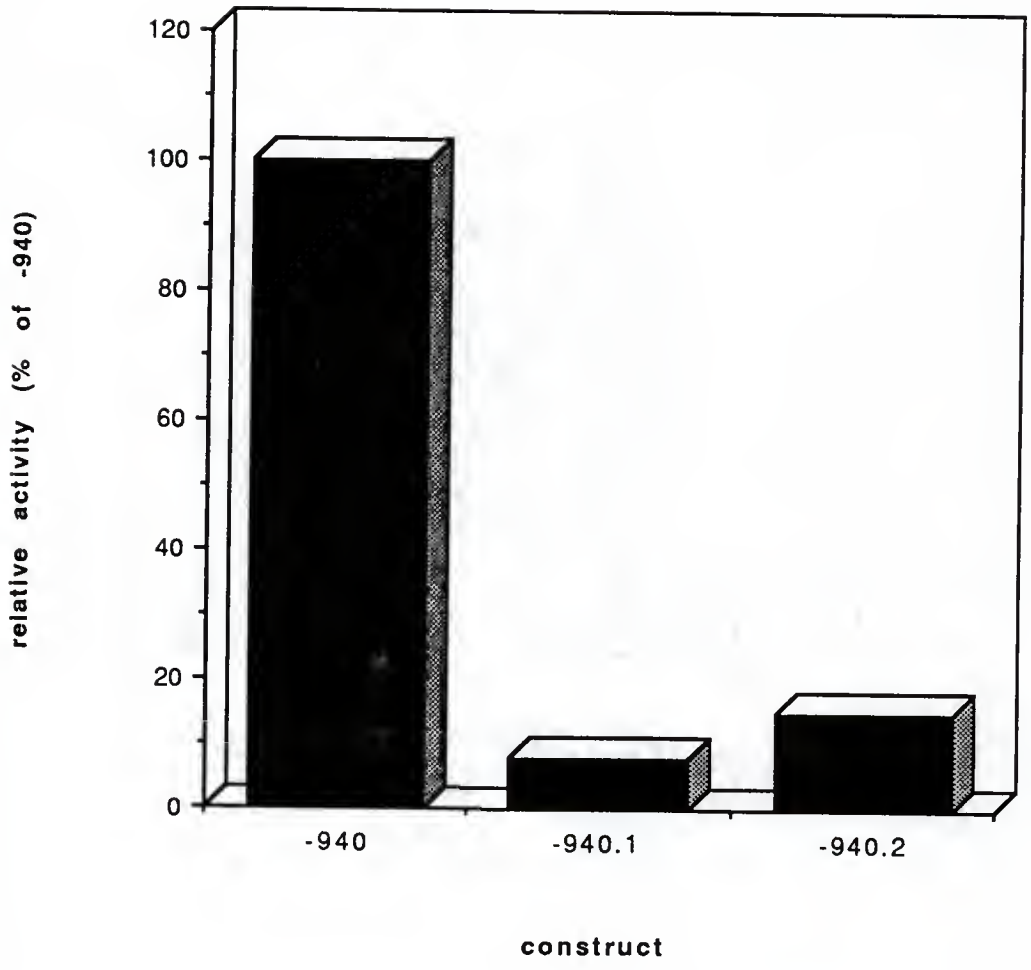


Figure 3-21. GUS activities of G-box Mutation Constructs relative to the -940/GUS.

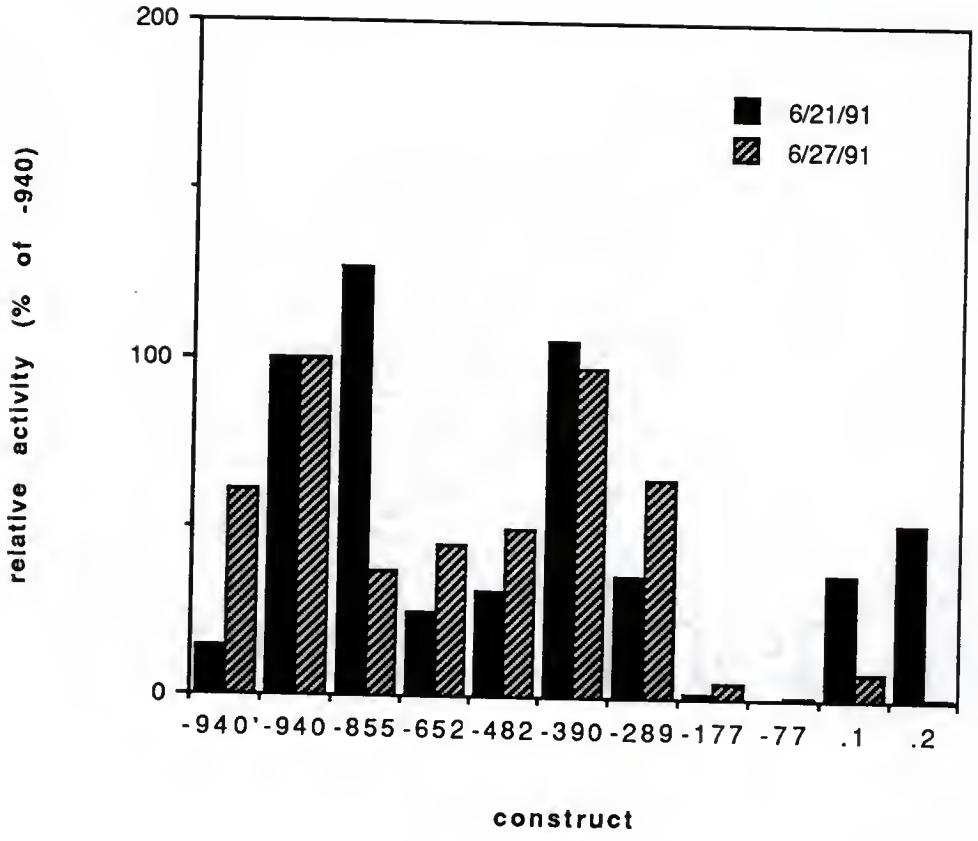


Figure 3-22. Comparison of Relative Gus activities between Experiments 6/21/91 and 6/27/91.

constructs were not observed to fall into obvious groupings. Most notably, the activity of -855/GUS in the experiment of 6/27/91 is significantly reduced in comparison to the full length construct. Furthermore, the G-box mutant constructs were reduced in activity compared to the experiment of 6/21/91, and are now significantly less active than the -652, -482, and -289 deletions. The relative activities of -390/GUS, -177/GUS and -77/GUS, however, were similar to those obtained in the experiment of 6/21/91 (Figure 3-22).

In order to reduce variability, a third experiment was designed which included 3 replicates for each construct. The large number of transformations required for this experiment necessitated the elimination of a few co nstructs due to protoplast yield and time limitations, so the G-box mutations were not examined here. All other experimental conditions were unchanged.

The results of this experiment, 7/11/90, are shown in Tables 3-7 and 3-8, and the data are plotted in Figures 3-23, 3-24, 3-25, 3-26, and 3-27. The slopes for each treatment were averaged, and their means and standard deviations are shown in Table 3-9. These results are presented graphically in Figure 3-28.

GUS activity of the deletion constructs from -940/GUS to -390/GUS appears to vary considerably. Closer inspection of these averages and their correspond-

Table 3-7

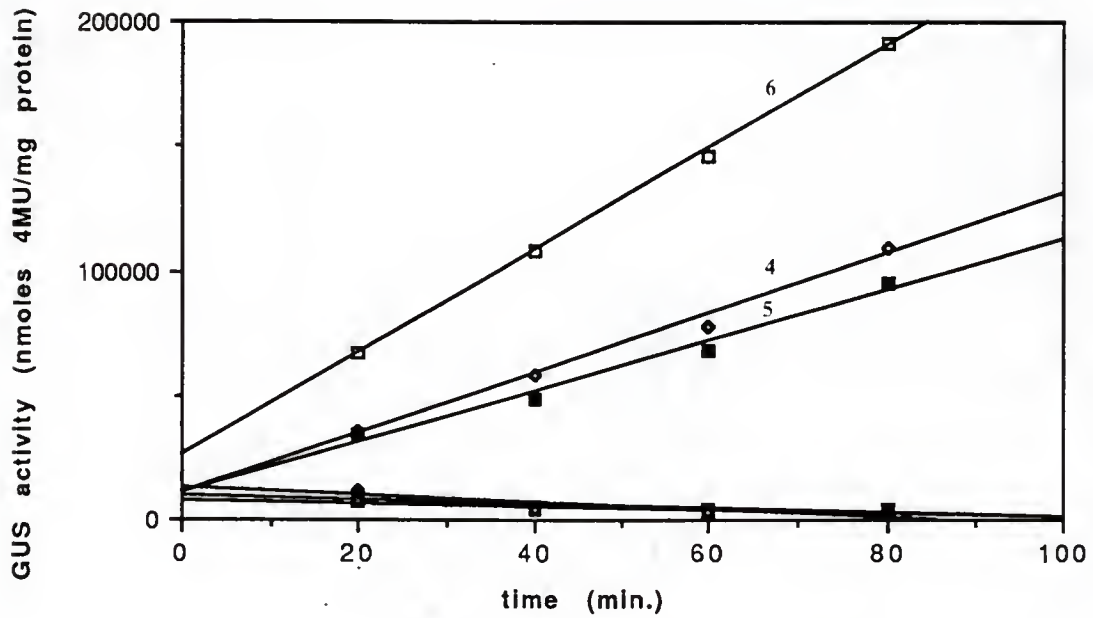
Total Protein and Fluorescence for Experiment 7/11/91

	total protein (ug/ul)	Fluorescence (nM 4MU) 20'	40'	60'	80'
1	0.351	0.130	0.072	0.058	0.058
2	0.294	0.138	0.065	0.029	0.033
3	0.341	0.097	0.061	0.047	0.045
4	0.312	0.445	0.729	0.968	1.361
5	0.286	0.393	0.561	0.782	1.089
6	0.227	0.611	0.986	1.329	1.739
7	0.275	0.434	0.497	0.686	0.867
8	0.327	0.348	0.576	0.783	1.030
9	0.310	0.417	0.743	1.060	1.457
10	0.272	0.413	0.606	0.784	0.986
11	0.315	0.361	0.572	0.750	0.877
12	0.259	0.285	0.387	0.486	0.665
13	0.339	0.369	0.566	0.780	1.004
14	0.283	0.275	0.505	0.695	0.969
15	0.308	0.297	0.610	0.725	0.958
16	0.313	0.314	0.606	0.867	1.133
17	0.292	0.266	0.533	0.795	1.031
18	0.339	0.395	0.814	1.412	1.498
19	0.274	0.478	1.057	1.548	1.947
20	0.251	0.342	0.755	1.017	1.362
21	0.254	0.426	0.738	1.149	1.515
22	0.258	0.237	0.438	0.716	0.808
23	0.348	0.245	0.551	0.701	1.037
24	0.310	0.200	0.366	0.585	0.657
25	0.353	0.083	0.140	0.167	0.143
26	0.353	0.061	0.078	0.090	0.096
27	0.297	0.091	0.102	0.130	0.119
28	0.297	0.075	0.069	0.049	0.032
29	0.392	0.070	0.074	0.039	0.044
30	0.298	0.078	0.071	0.069	0.059

Table 3-8

Protein per Assay and Fluorescence per mg Protein for Experiment 7/11/91

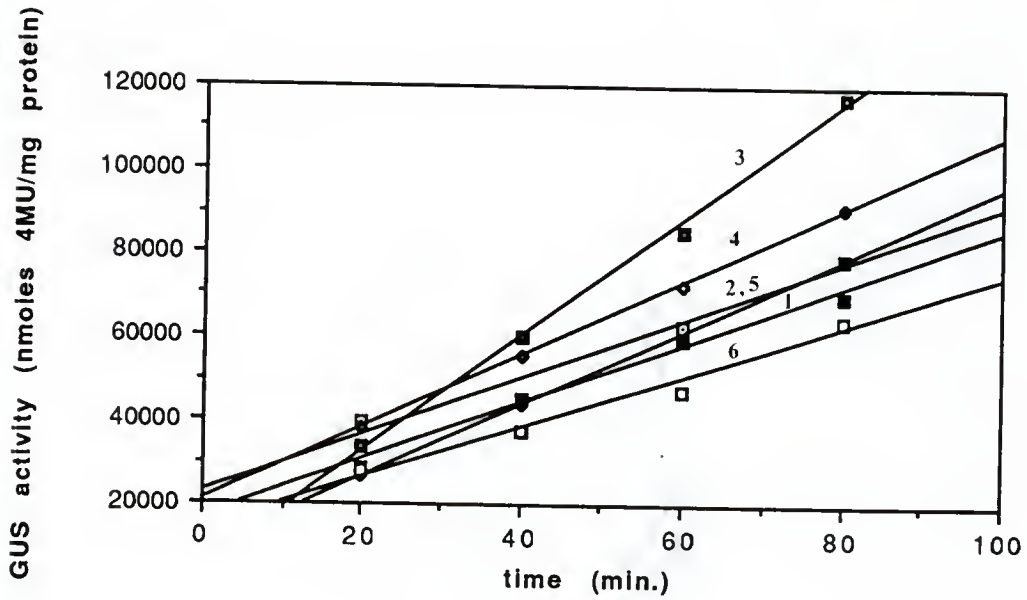
	protein per assay (mg x 10^{-5})	Corrected Fluorescence (nmoles 4MU/mg protein) 20'	40'	60'	80'
1	1.40	9286	5142	4142	
2	1.18	11744	5531	2468	2808
3	1.36	7132	4472	3445	3299
4	1.25	35686	58460	77626	109141
5	1.14	34413	49124	68476	95359
6	0.91	67201	108446	146172	191267
7	1.10	39526	45264	62477	78961
8	1.31	26605	44036	59862	78746
9	1.24	33574	59822	85346	117340
10	1.09	38029	55801	72191	90791
11	1.26	28696	45468	59618	69713
12	1.04	27483	37319	46865	64127
13	1.36	27172	41679	57437	73932
14	1.13	24314	44650	61450	85676
15	1.23	24146	49593	58943	77235
16	1.25	25059	48363	69193	90422
17	1.17	22735	45555	67948	88119
18	1.36	29086	59941	103976	110309
19	1.10	43533	96265	140983	177322
20	1.00	34362	75199	101294	135657
21	1.01	42011	72781	113313	149408
22	1.03	22987	42483	69447	78370
23	1.39	17613	39611	50395	74550
24	1.24	16103	29468	47101	52898
25	1.41	5874	9907	11818	10120
26	1.41	4317	5520	6369	6794
27	1.19	7666	8593	10951	10025
28	1.19	6318	5812	4128	2695
29	1.57	4464	4719	2487	2806
30	1.19	6543	5956	5788	4949



1	no DNA	y = 9786.0 - 82.160x	R^2 = 0.749
2	no DNA	y = 1.3106e+4 - 149.35x	R^2 = 0.806
3	no DNA	y = 7718.5 - 62.630x	R^2 = 0.830
4	-940/GUS*	y = 1.0346e+4 + 1197.7x	R^2 = 0.989
5	-940/GUS*	y = 1.1296e+4 + 1010.9x	R^2 = 0.982
6	-940/GUS*	y = 2.5791e+4 + 2049.6x	R^2 = 0.999

Figure 3-23. Time course of Fluorescence from Experiment 7/11/91.

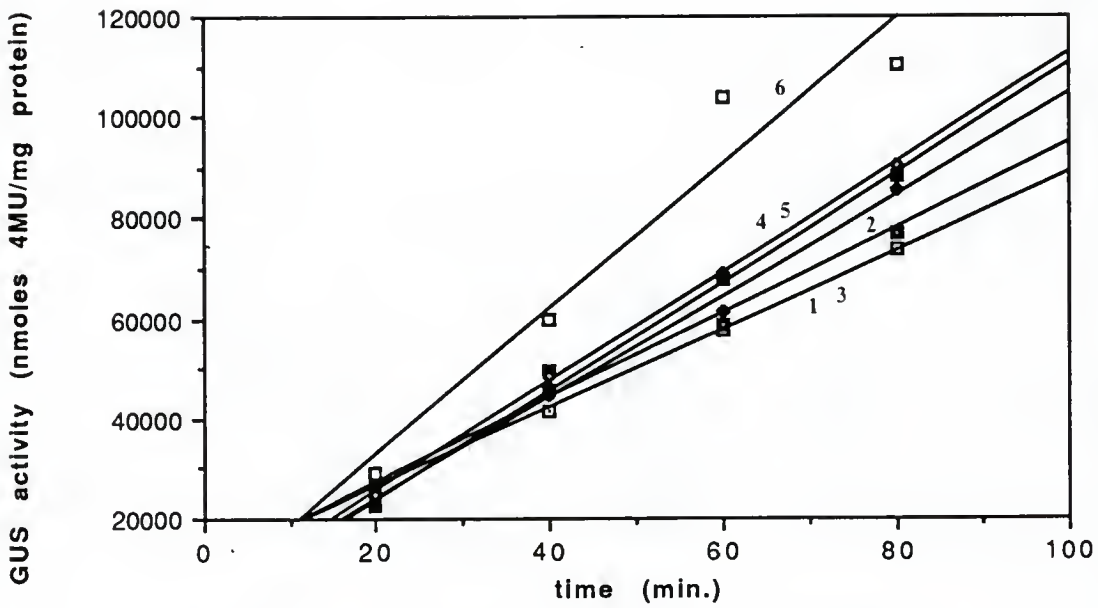
All treatments in this were done in triplicate. Above are shown the no DNA control vs. -940/GUS with no plasmid carrier DNA.



1	-940/GUS	y = 2.2678e+4 + 677.59x	R^2 = 0.962
2	-940/GUS	y = 9250.0 + 861.23x	R^2 = 0.999
3	-940/GUS	y = 4815.0 + 1384.1x	R^2 = 0.997
4	-855/GUS	y = 2.0534e+4 + 873.38x	R^2 = 0.999
5	-855/GUS	y = 1.6574e+4 + 686.00x	R^2 = 0.988
6	-855/GUS	y = 1.4079e+4 + 597.39x	R^2 = 0.977

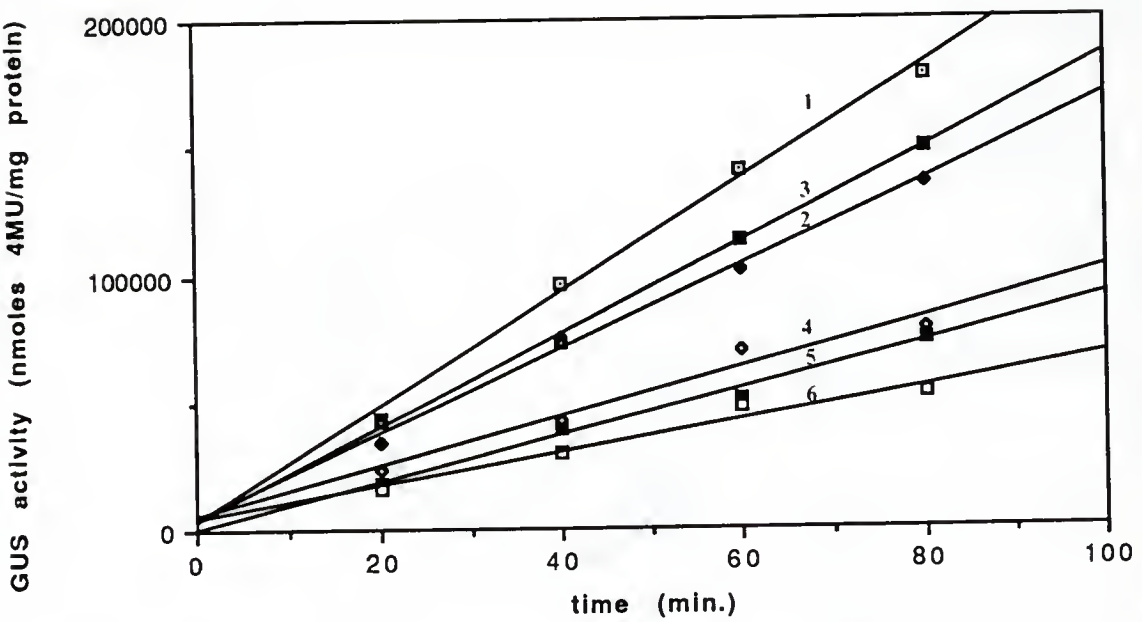
Figure 3-24. Time course of Fluorescence from Experiment 7/11/91.

The above treatments contain carrier DNA, as do all remaining treatments.



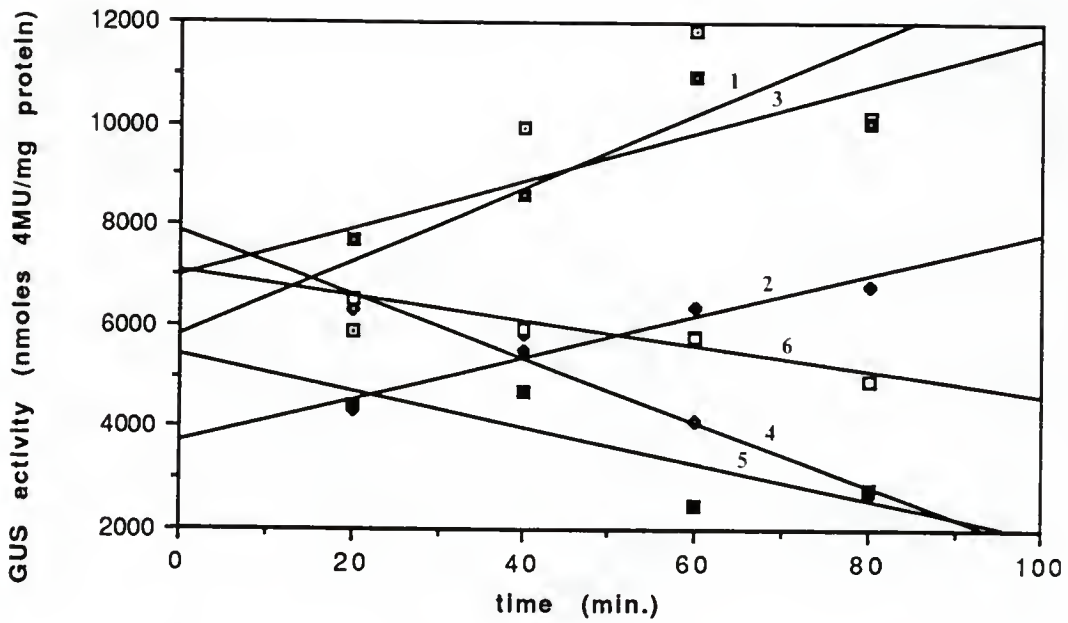
1	-652/GUS	y = 1.1046e+4 + 780.19x	R^2 = 0.999
2	-652/GUS	y = 3801.0 + 1004.4x	R^2 = 0.995
3	-652/GUS	y = 1.0325e+4 + 843.08x	R^2 = 0.970
4	-482/GUS	y = 4029.5 + 1084.6x	R^2 = 0.999
5	-482/GUS	y = 1453.0 + 1092.7x	R^2 = 0.999
6	-482/GUS	y = 3902.0 + 1438.5x	R^2 = 0.937

Figure 3-25. Time course of Fluorescence from Experiment 7/11/91.



1	▫	-390/GUS	y = 3004.5 + 2230.4x	R^2 = 0.993
2	◆	-390/GUS	y = 4133.0 + 1649.9x	R^2 = 0.993
3	■	-390/GUS	y = 3697.5 + 1813.6x	R^2 = 0.997
4	◇	-289/GUS	y = 5043.5 + 965.56x	R^2 = 0.969
5	■	-289/GUS	y = 143.50 + 907.97x	R^2 = 0.981
6	□	-289/GUS	y = 4388.0 + 640.09x	R^2 = 0.968

Figure 3-26. Time course of Fluorescence from Experiment 7/11/91.



1 ◻	-177/GUS	y = 5767.5 + 73.245x	R^2 = 0.563
2 ◆	-177/GUS	y = 3680.0 + 41.400x	R^2 = 0.958
3 ■	-177/GUS	y = 6950.0 + 47.175x	R^2 = 0.693
4 ◆	-77/GUS	y = 7876.5 - 62.765x	R^2 = 0.961
5 ■	-77/GUS	y = 5420.5 - 36.030x	R^2 = 0.672
6 ◻	-77/GUS	y = 7046.5 - 24.750x	R^2 = 0.942

Figure 3-27. Time course of Fluorescence from Experiment 7/11/91.

Table 3-9.

GUS Activities, Means, and Standard Deviations from Experiment 7/11/91.

GUS activity (nmoles/min/mg)	mean	SD
-82		
-149		
-62		
1197		
1010		
2049	1418	553
678		
861		
1384	974	366
873		
686		
597	718	140
780		
1004		
843	875	115
1084		
1092		
1438	1204	202
2230		
1649		
1813	1897	299
965		
907		
640	837	173
73		
41		
47	53	17
-62		
-36		
-24		

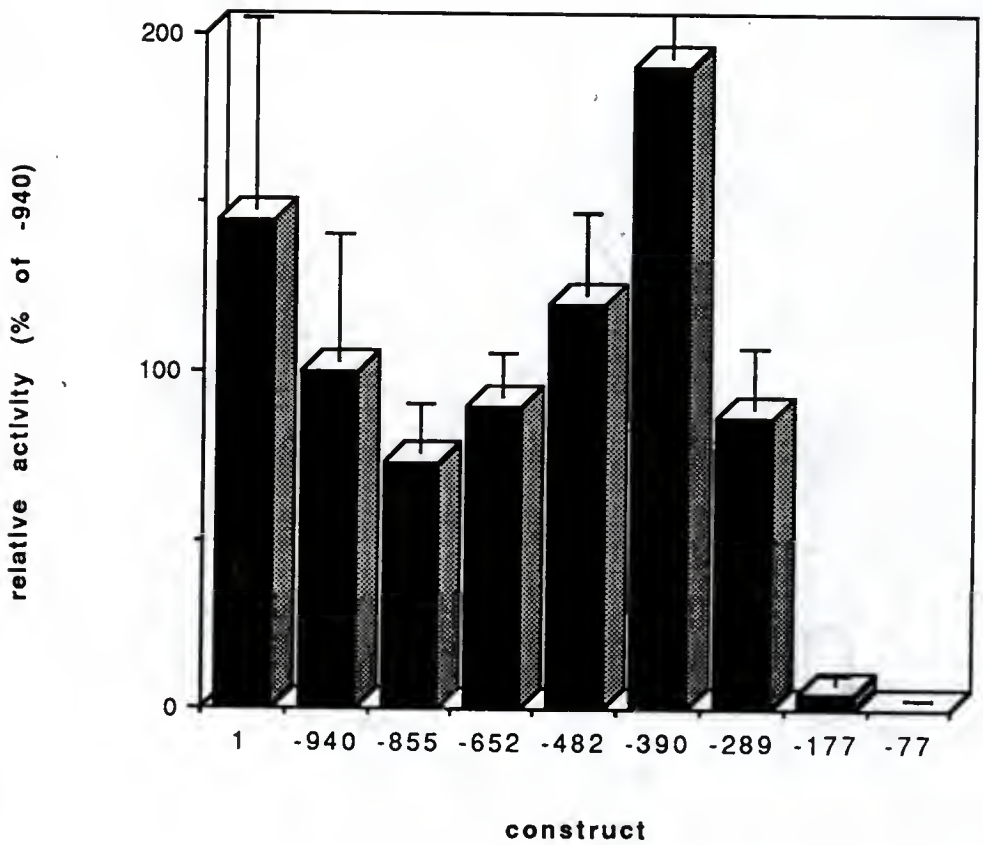


Figure 3-28. GUS activities of Deletion Constructs Relative to -940/GUS.

Activities are reported as a percentage of -940/GUS. Error bars are +/- standard deviation. Column 1 represents a -940/GUS transformation without carrier DNA.

ing error rates, however, reveals no statistical difference between the full length promoter (-940/GUS), and -855/GUS, -652/GUS, and -482/GUS. The -390/GUS construct is more active than the full length promoter by approximately 20%, yet even this difference does not withstand rigorous statistical analysis (students t-test). A statistically significant difference may be observed, however, between -390/GUS, -289/GUS, and -177/GUS. The -289/GUS construct is roughly 50% the activity of -390/GUS, and further deletion to -177 reduces GUS activity to less than 10%.

The variation in activity among the TATA distal 5' deletion constructs -940/GUS, -855/GUS, -652/GUS, -482/GUS, and -390/GUS has apparently not been eliminated by replication of treatment. It is interesting to note, however, that the TATA proximal deletions -390/GUS, -289/GUS, -177/GUS, and -77/GUS behave with relative consistency among the three experiments. In order to reduce variation among the distal constructs, the following experiment was designed to reduce error arising from variation in transformation efficiency.

An alternate reference gene construct (35S/Luciferase) was included in the following experiment in replacement of -940/CAT. The buffer used to extract GUS does not preserve luciferase activity, and this required splitting all samples in half and resuspending cells in

different buffers prior to extraction. This resulted in decreased protein per assay, and time points for the GUS assay were reduced by half in order to remain within the linear range of GUS activity.

Assay of all constructs by this method was not possible in a single experiment, and required 3 separate experiments for completion. The first of these is experiment 8/23/91, which tested the activities of -855/GUS, -652/GUS, -482/GUS, and -390/GUS relative to -940/GUS. The results of this experiment for GUS activity are shown in Tables 3-10 and 3-11, and plotted in Figures 3-29 and 3-30, and 3-31. Corresponding protein and luminescence (in relative light units, or RLU) values are shown in Table 3-12. The GUS activities derived from Figures 3-29, 3-30, and 3-31 were corrected for transformation efficiency using corresponding luciferase levels, and replicate activities averaged as shown in Table 3-13.

The second of this series of experiments (9/17/91) included the G-box mutant constructs -940.1/GUS and -940.2/GUS in addition to the deletion constructs -289/GUS, -177/GUS, -77/GUS, and a repeat of -652/GUS. The results for GUS activity are presented in Table 3-14 and 3-15, and plotted in Figures 3-32, 3-33, and 3-34. Corresponding RLUs and associated protein levels are shown in Table 3-16, and corrected GUS activities and their means are shown in table 3-17.

TABLE 3-10

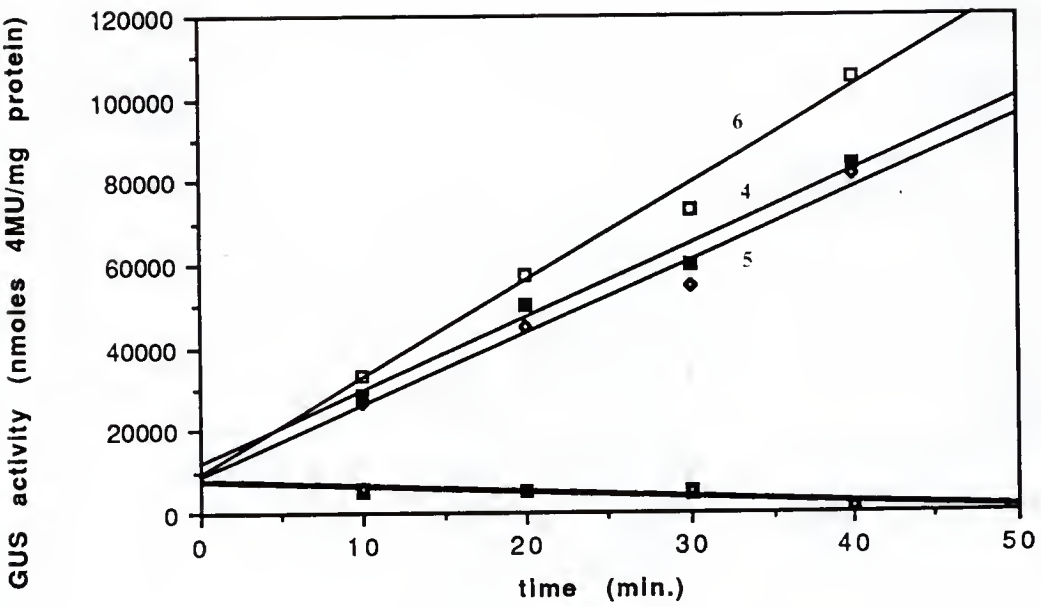
Total Protein and Fluorescence for Experiment 8/23/91

	total protein (ug/ul)	Fluorescence (nM 4MU) 10'	20'	30'	40'
1	0.830	0.835	0.851	0.780	0.188
2	0.718	0.797	0.768	0.748	0.178
3	0.709	0.805	0.776	0.748	0.162
4	0.521	2.771	4.628	5.624	8.388
5	0.560	3.158	5.584	6.662	9.342
6	0.589	3.849	6.699	8.546	12.26
7	0.412	3.518	5.885	7.770	9.648
8	0.379	5.549	6.101	7.756	10.49
9	0.517	4.297	7.841	9.188	13.72
10	0.508	0.801	0.750	0.108	0.476
11	0.516	0.800	0.788	0.104	0.276
12	0.511	0.775	0.776	0.110	0.206
13	0.622	3.795	6.580	8.410	11.96
14	0.639	4.908	8.907	12.01	16.78
15	0.647	5.408	9.773	13.83	18.79
16	0.678	3.456	6.131	7.704	11.60
17	0.652	3.056	5.271	6.644	9.068
18	0.549	2.616	4.286	5.130	7.248

TABLE 3-11

Protein per assay and Fluorescence per mg protein for Experiment 8/23/91

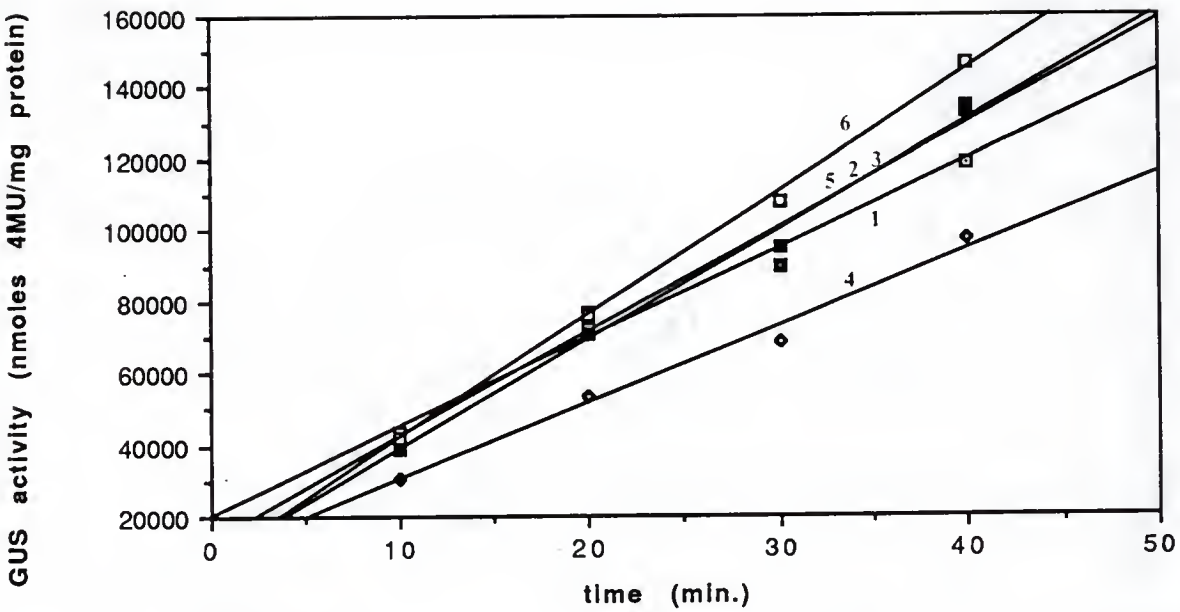
	protein per assay ($mgx10^{-4}$)	Corrected Fluorescence (nmoles 4MU/mg protein) 10	20	30	40
1	1.64	5091	5189	4756	1146
2	1.42	5605	5401	5260	1251
3	1.40	5738	5531	5331	1154
4	1.03	26903	44932	54601	81436
5	1.11	28476	50352	60070	84238
6	1.12	33011	57453	73293	105197
7	0.82	43096	72094	95190	118191
8	1.15	30969	53237	64180	91570
9	1.02	42004	76647	89800	134096
10	1.23	30828	53452	68318	97189
11	1.27	38798	70411	94900	132640
12	1.28	42217	76291	107900	146740
13	1.34	25772	45719	57450	86517
15	1.29	23689	40860	51500	70290
16	1.09	24089	39466	47250	66700



1 ▫	no DNA	y = 7112.5 - 122.68x	R^2 = 0.665
2 ◆	no DNA	y = 7680.0 - 132.03x	R^2 = 0.665
3 ■	no DNA	y = 7926.5 - 139.52x	R^2 = 0.673
4 ◆	-940/GUS	y = 8651.0 + 1732.7x	R^2 = 0.967
5 ■	-940/GUS	y = 1.1533e+4 + 1770.0x	R^2 = 0.977
6 ▫	-940/GUS	y = 9139.0 + 2324.0x	R^2 = 0.984

Figure 3-29. Time course of Fluorescence for Experiment 8/23/91.

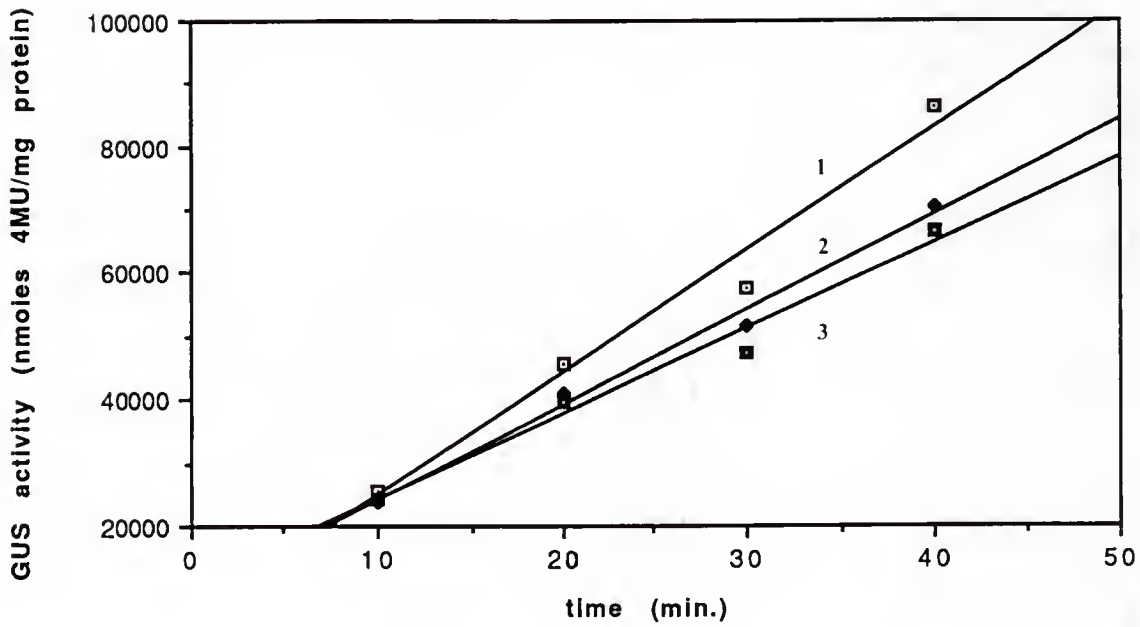
All conditions are as in Experiment 7/11/91 with the exception of 35S/Luciferase taking the place of 35S/CAT as the reference construct.



1	□	-855/GUS	y = 2.0048e+4 + 2483.8x	R^2 = 0.997
2	◆	-855/GUS	y = 1.3280e+4 + 2894.3x	R^2 = 0.963
3	■	-855/GUS	y = 1.3280e+4 + 2894.3x	R^2 = 0.963
4	◆	-482/GUS	y = 8959.5 + 2139.5x	R^2 = 0.986
5	■	-482/GUS	y = 7683.5 + 3060.1x	R^2 = 0.994
6	□	-482/GUS	y = 6992.5 + 3451.8x	R^2 = 0.998

Figure 3-30. Time course of Fluorescence From Experiment 8/23/91.

The -652/GUS construct (lines 10, 11, and 12 from Table 3-10) did not produce detectable fluorescence and is omitted.



1 ▫ -390/GUS y = 5373.0 + 1939.7x R^2 = 0.972
2 ◆ -390/GUS y = 8974.0 + 1504.4x R^2 = 0.990
3 ▪ -390/GUS y = 1.0472e+4 + 1356.2x R^2 = 0.976

Figure 3-31. Time course of Fluorescence for Experiment 8/23/91.

Table 3-12

Protein and Relative Light Units for Experiment 8/23/91

	total protein (ug/10ul)	protein per assay ($mgx10^{-4}$)	RLU	$mgx10^4$	RLU per
1	0.494	9.89		825	83
2	0.383	7.66		702	91
3	0.281	5.62		574	102
4	0.223	4.47		337119	75485
5	0.234	4.67		370092	79181
6	0.099	1.97		53680	27193
7	0.215	4.30		431523	100449
8	0.226	4.52		626739	138536
9	0.178	3.56		485745	136445
10	0.219	4.37		442641	101244
11	0.267	5.34		638643	119685
12	0.204	4.07		612067	150385
13	0.214	4.28		288633	67476
14	0.208	4.16		287104	68949
15	0.203	4.05		376357	92881

Table 3-13

Correcting GUS activity for transformation efficiency using Luciferase for experiment 8/23/91

	GUS activity (pmol/min/mg)	RLU (mgx10^4)	GUS/RLU (10^5)	MEAN	SD
1	1733	75485	2296	2265*	35
2	1770	79181	2235		
3	2324	27193	8550		
4	2484	100447	2473	2026	565
5	1928	138536	1392		
6	3029	136445	2220		
7	2140	101244	2113	2320	226
8	3060	119685	2557		
9	3452	150385	2295		
10	1940	67476	2875	2170	705
11	1504	68949	2182		
12	1356	92881	1460		

TABLE 3-14

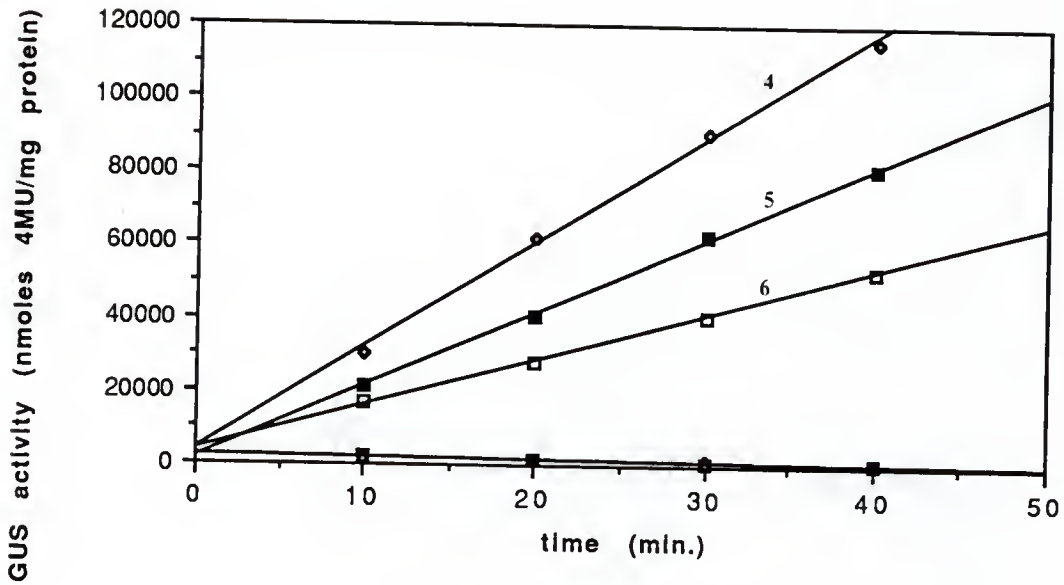
Total Protein and Fluorescence for Experiment 9/17/91

	total protein (ug/10ul)	Fluorescence (nM 4MU) 10'	20'	30'	40'
1	0.386	0.179	0.154	0.092	0.051
2	0.380	0.177	0.138	0.104	0.051
3	0.449	0.198	0.153	0.109	0.068
4	0.352	2.644	5.370	7.889	10.04
5	0.274	1.466	2.723	4.220	5.478
6	0.163	0.671	1.127	1.645	2.150
7	0.267	1.529	2.777	4.252	5.643
8	0.249	0.727	1.172	1.763	2.222
9	0.292	0.780	1.369	1.935	2.506
10	0.263	0.920	1.737	2.591	3.357
11	0.218	0.712	1.213	1.826	2.368
12	0.212	0.771	1.321	1.972	2.621
13	0.268	0.173	0.268	0.099	0.070
14	0.278	0.149	0.105	0.081	0.053
15	0.267	0.162	0.099	0.080	0.043
16	0.159	0.317	0.396	0.551	0.684
17	0.236	1.407	2.665	3.917	5.224
18	0.170	0.475	0.768	1.162	1.441
19	0.218	0.186	0.284	0.232	0.284
20	0.229	0.172	0.164	0.176	0.177
21	0.191	0.168	0.173	0.165	0.168
22	0.221	0.266	0.099	0.055	0.062
23	0.225	0.135	0.094	0.042	0.055
24	0.145	0.160	0.089	0.059	0.053

Table 3-15

Protein per assay and Fluorescence per mg protein for experiment 9/17/91

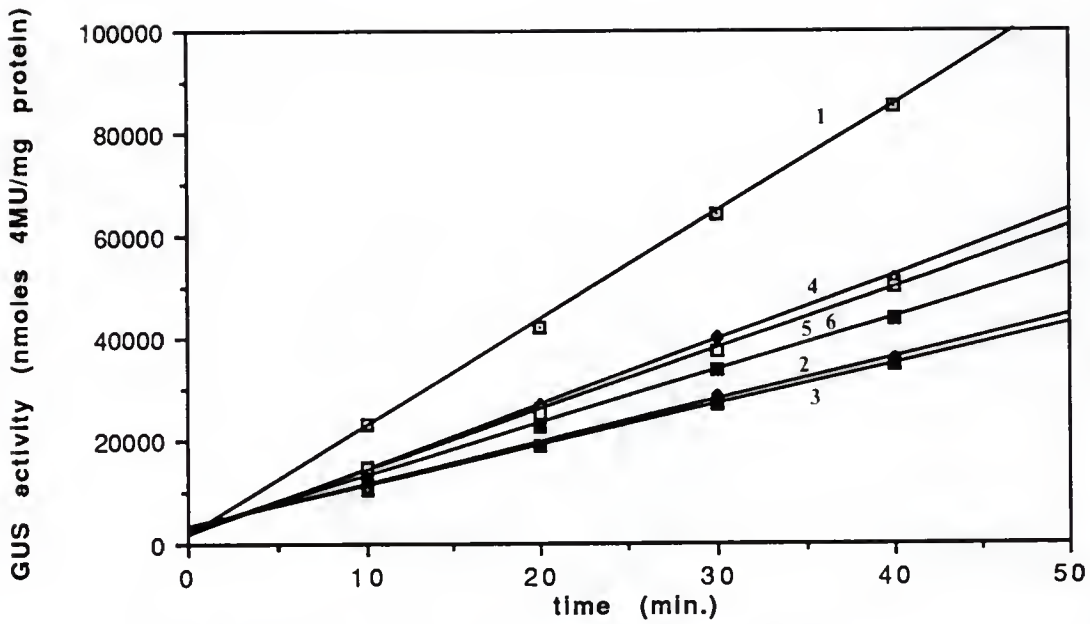
	protein per assay ($mgx10^{-3}$)	Corrected Fluorescence (nmoles 4MU/mg protein) 10'	20'	30'	40'
1	9.55	1874	1612	963	533
2	9.41	1881	1467	1105	542
3	11.1	1781	1376	980	612
4	8.72	30338	61618	90520	115249
5	6.78	21625	40168	62250	80808
6	4.03	16645	27958	40810	53337
7	6.60	23156	42057	64395	85461
8	6.17	11783	18995	28573	36013
9	7.24	10774	18911	26730	34618
10	6.52	14117	26653	39760	51511
11	5.40	13209	22504	33870	43930
12	5.25	14697	25181	37590	49960
13	3.94	4599	10040	13970	17342
14	5.85	24039	45536	66920	89253
15	4.20	11298	18268	27640	34276
16	5.39	3450	5269	4304	5269
17	5.68	3029	2888	3099	3117
18	4.72	3556	3641	3493	3553
19	5.48	4856	1807	1004	1132
20	5.56	2426	1689	7586	988
21	3.60	4448	2474	1640	1473



1 ◻	no DNA	y = 2413.5 - 46.720x	R^2 = 0.977
2 ◆	no DNA	y = 2343.5 - 43.790x	R^2 = 0.991
3 ■	no DNA	y = 2163.0 - 39.030x	R^2 = 1.000
4 ◆	-940/GUS	y = 3522.5 + 2836.3x	R^2 = 0.997
5 ■	-940/GUS	y = 1305.0 + 1996.3x	R^2 = 0.999
6 ◻	-940/GUS	y = 3955.5 + 1229.3x	R^2 = 0.999

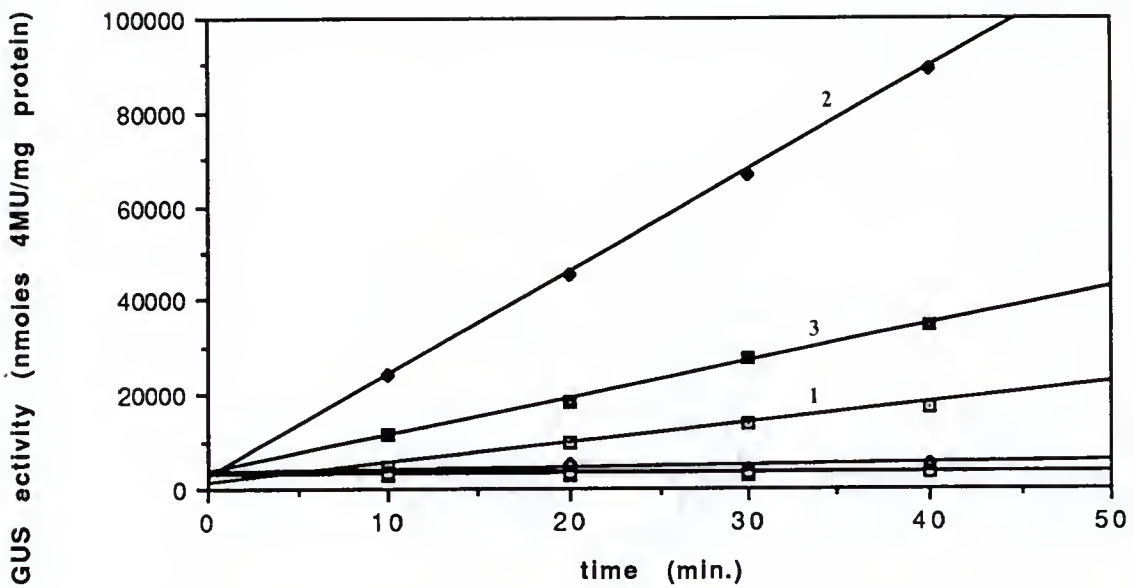
Figure 3-31. Time course of Fluorescence from Experiment 9/17/91.

Experimental conditions are identical to those of experiment 8/23/91.



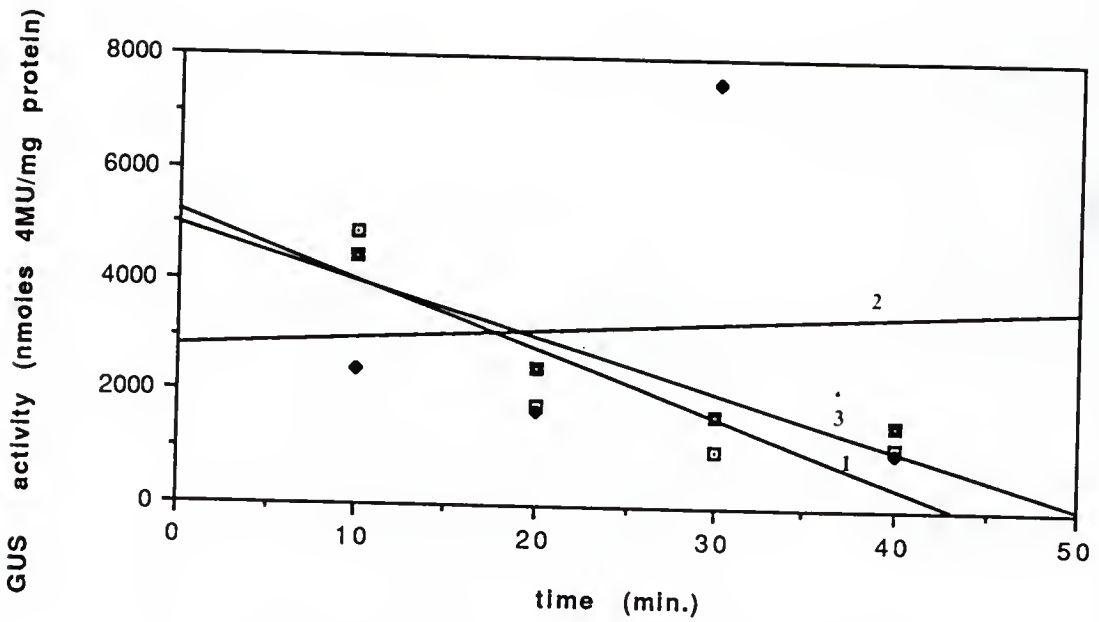
1 ◻	-940.1/GUS	y = 1454.0 + 2092.5x	R^2 = 0.999
2 ◆	-940.1/GUS	y = 3274.0 + 822.68x	R^2 = 0.997
3 ■	-940.1/GUS	y = 2920.5 + 793.51x	R^2 = 1.000
4 ◆	-940.2/GUS	y = 1688.0 + 1252.9x	R^2 = 1.000
5 ■	-940.2/GUS	y = 2496.0 + 1035.3x	R^2 = 0.999
6 ◻	-940.2/GUS	y = 2307.5 + 1182.0x	R^2 = 0.998

Figure 3-32. Time course of Fluorescence from Experiment 9/71/91.



1	□	-289/GUS	y = 948.00 + 421.59x	R^2 = 0.988
2	◆	-289/GUS	y = 2180.5 + 2170.3x	R^2 = 1.000
3	■	-289/GUS	y = 3294.0 + 783.06x	R^2 = 0.996
4	◆	-177/GUS	y = 3450.0 + 44.920x	R^2 = 0.438
5	■	-177/GUS	y = 2884.5 + 6.5500x	R^2 = 0.475
6	□	-177/GUS	y = 3600.0 - 1.5700x	R^2 = 0.111

Figure 3-33. Time course of Fluorescence from Experiment 9/17/91.



1	-77/GUS	y = 5193.5 - 119.75x	R^2 = 0.733
2	-77/GUS	y = 2776.5 + 15.830x	R^2 = 0.005
3	-77/GUS	y = 4948.5 - 97.590x	R^2 = 0.852

Figure 3-34. Time course of Fluorescence for Experiment 9/17/91.

TABLE 3-16

Protein and RLU for Experiment 9/18/91

	total protein (ug/10ul)	protein/ assay	RLU	RLU/ (mgx10^4)
1	0.169	3.38	868	257
2	0.169	3.38	689	204
3	0.159	3.18	578	182
4	0.053	1.06	109101	102346
5	0.071	1.42	121974	86261
6	0.046	0.92	50219	54586
7	0.074	1.48	275539	185174
8	0.063	1.26	131632	105305
9	0.081	1.62	198504	122836
10	0.085	1.70	286315	169417
11	0.066	1.32	210388	158903
12	0.077	1.54	262996	170555
13	0.079	1.58	356	562
14	0.061	1.22	621	509
15	0.074	1.48	308	208
16	0.047	0.94	32595	34749
17	0.085	1.70	256314	149891
18	0.066	1.32	90197	68124
19	0.085	1.70	39269	82407
20	0.066	1.32	123143	93008
21	0.075	1.50	124278	82521
22	0.057	1.14	117339	102928
23	0.079	1.58	102338	64770
24	0.073	1.46	140506	96900

TABLE 3-17

GUS activity Corrected for Transformation efficiency with Luciferase

	GUS activity (pmole/min/mg)	RLU/ protein	Corrected GUS activity	MEAN	SD
1	2836	102346	2770		
2	1996	86261	2310		
3	1229	54586	2250	2443	284
4	2093	185174	1130		
5	822	105305	780		
6	793	122836	646	852	249
7	1253	169417	739		
8	1035	158903	652		
9	1182	175555	673	688	45
10	422	34749	1210		
11	2170	149891	1450		
12	783	68124	1150	1270	158
13	44	82407	54		
14	475	93008	510		
15	00	82521	00	188	280
16	00	102928	00		
17	00	64770	00		
18	00	96900	00	0	0

The -652/GUS construct, not having shown expression in the previous two experiments, was re-purified and transformed in the final experiment (9/27/91). GUS protein levels and fluorescence data are shown in Tables 3-18 and 3-19, and plotted in Figure 3-34. Corresponding protein and luciferase RLUs are shown in Table 3-20, and the corrected GUS activities and means in Table 3-21.

The results of experiments 8/23/91, 9/17/91, and 9/27/91 are presented in Figures 3-35 (deletion constructs) and 3-36 (G-box mutation constructs). The TATA distal deletion constructs -855/GUS, -652/GUS, -482/GUS, and -390/GUS all appeared to express GUS at levels equivalent to the full length promoter. Deletion to -289/GUS resulted in a decrease in activity of approximately 50%, and further deletion to -177/GUS reduced GUS expression to less than 10% of -940/GUS.

Table 3-18

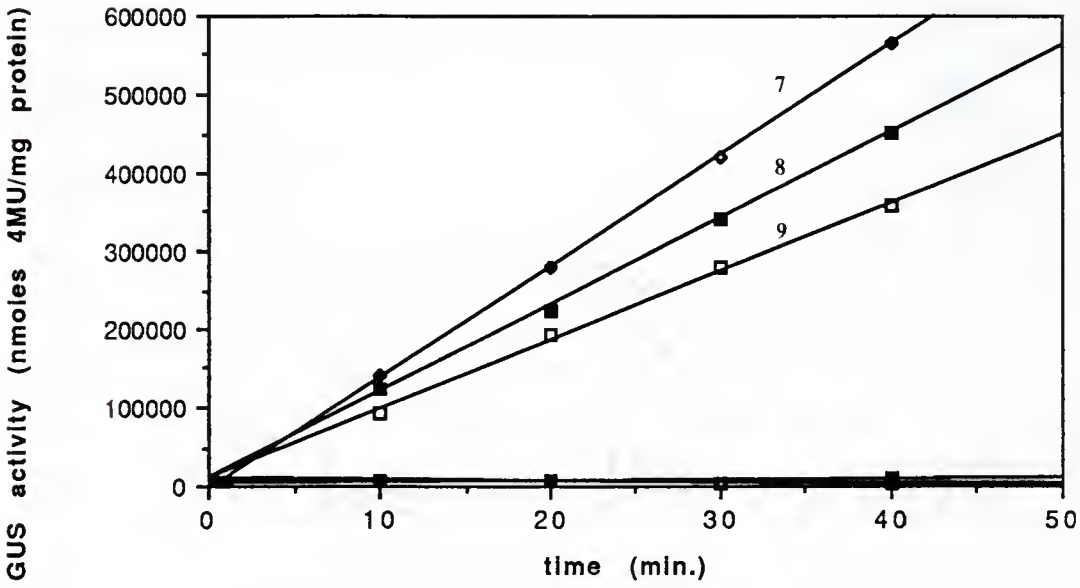
Total protein and Fluorescence for Experiment 9/27/91

	total protein (ug/10ul)	Fluorescence (nM 4MU) 10'	20'	30'	40'
1	0.434	0.149	0.096	0.082	0.054
2	0.434	0.102	0.091	0.088	0.061
3	0.374	0.100	0.087	0.074	0.164
4	0.288	1.619	3.203	4.865	6.532
5	0.325	1.595	2.927	4.434	5.869
6	0.276	1.025	2.116	3.074	3.957
7	0.391	3.244	6.295	9.460	11.94
8	0.376	2.889	5.880	8.230	10.66
9	0.331	2.912	5.095	7.075	9.644

TABLE 3-19

Protein per Assay and Fluorescence per mg protein for Experiment 9/27/91

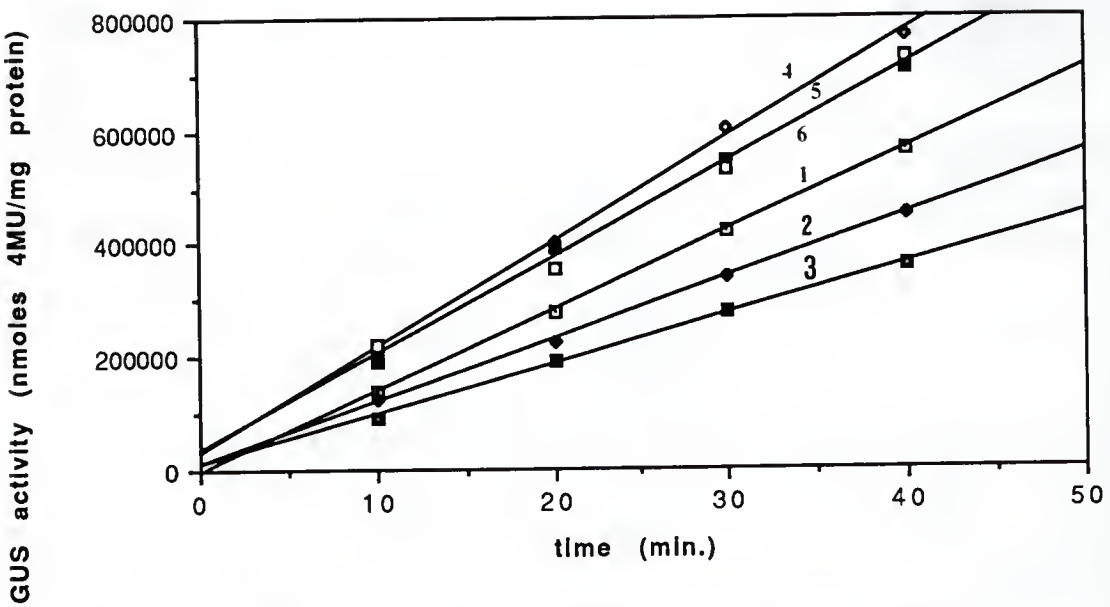
	protein per assay ($mgx10^{-3}$)	Corrected Fluorescence (nmoles 4MU/mg protein) 10'	20'	30'	40'
1	1.74	8500	5530	4720	3110
2	1.74	6830	5240	5070	3510
3	1.49	6690	5823	4950	10970
4	1.15	140538	278000	422300	567000
5	1.30	122690	225100	341000	451500
6	1.11	92760	191400	278100	358100
7	1.56	207550	402700	605200	764200
8	1.50	192340	391500	547900	709700
9	1.33	219700	355300	534000	727800



1	no DNA	y = 9710.0 - 169.80x	R^2 = 0.941
5	no DNA	y = 7695.0 - 101.30x	R^2 = 0.929
6	no DNA	y = 4116.5 + 119.67x	R^2 = 0.335
7	-940/GUS	y = - 3962.0 + 1.4237e+4x	R^2 = 1.000
8	-940/GUS	y = 9490.0 + 1.1023e+4x	R^2 = 0.999
9	-940/GUS	y = 9410.0 + 8827.2x	R^2 = 0.998

Figure 3-35. Time course of Fluorescence from Experiment 9/27/91.

All condition are identical to experiment 9/17/91.



1	-940/GUS	y = - 3962.0 + 1.4237e+4x	R^2 = 1.000
2	-940/GUS	y = 9490.0 + 1.1023e+4x	R^2 = 0.999
3	-940/GUS	y = 9410.0 + 8827.2x	R^2 = 0.998
4	-652/GUS	y = 2.6800e+4 + 1.8724e+4x	R^2 = 0.997
5	-652/GUS	y = 3.3240e+4 + 1.7085e+4x	R^2 = 0.997
6	-652/GUS	y = 3.3450e+4 + 1.7030e+4x	R^2 = 0.994

Figure 3-36. Time course of Fluorescence for Deletion Construct -652/GUS from Experiment 9/27/91.

Table 3-20

Total protein and RLU for experiment 9/27/91

	total protein (ug/10ul)	protein/ assay ($mgx10^{-4}$)	RLU	RLU/protein ($mgx10^{4}$)
1	0.296	5.92	637	108
2	0.315	6.30	526	83
3	0.289	5.78	435	75
4	0.230	4.60	209465	45536
5	0.127	2.55	161379	63286
6	0.230	4.60	158826	34527
7	0.199	3.97	245080	61733
8	0.202	4.04	307718	76168
9	0.190	3.79	339385	89547

Table 3-21

Corrected GUS activity for experiment 9/27/91

	GUS activity (nM/min/mg)	RLU/ protein	Corrected GUS	mean	SD
1	142.4	45536	3127		
2	110.2	63535	1734		
3	88.3	34527	2558	2472	700
4	187.2	61733	3029		
5	170.9	76167	2243		
6	167.4	89547	1869	2380	592

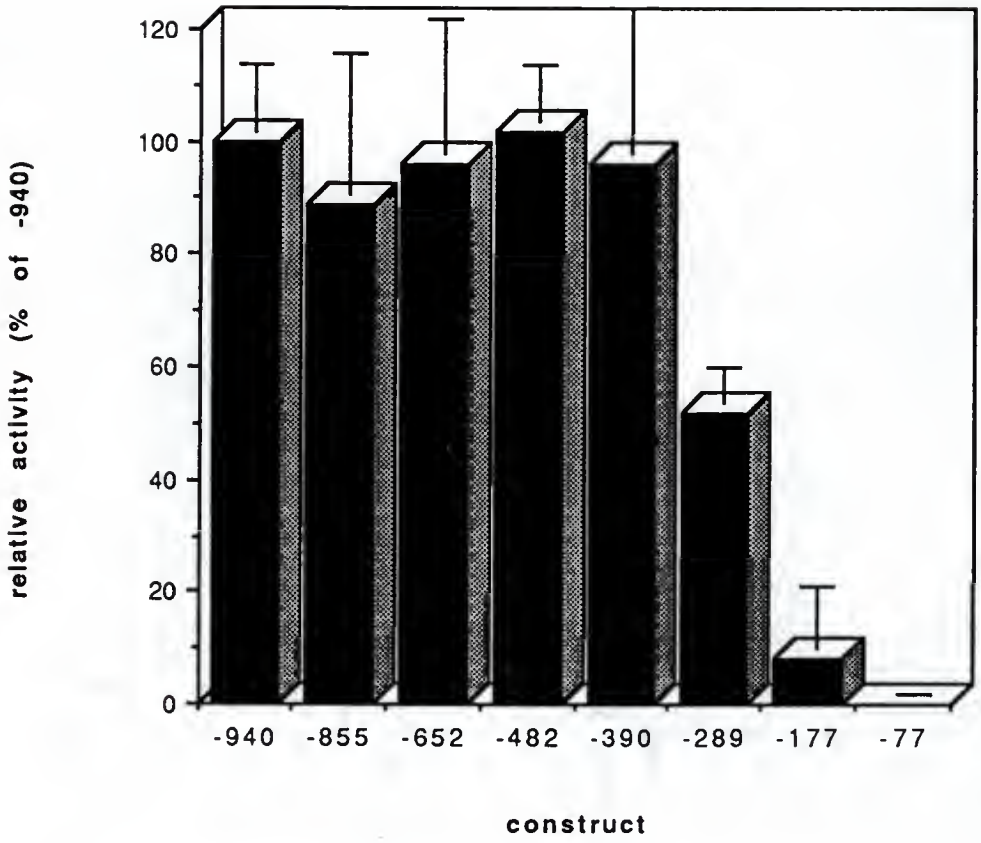


Figure 3-37. GUS activities of Deletion Constructs Relative to -940/GUS.

Relative activity is plotted as a percent of -940/GUS from the same experiment. This plot combines the results of the last three experiments (8/23/91, 9/17/91, and 9/27/91).

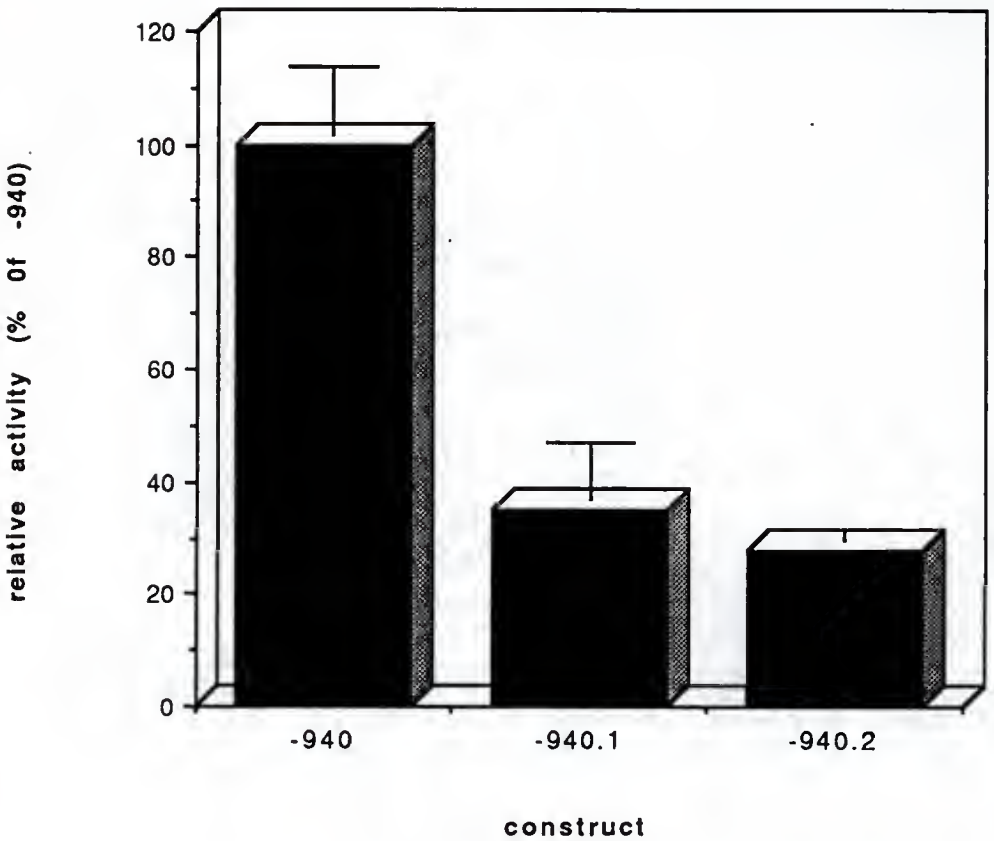


Figure 3-36. GUS activities of G-box Mutant Constructs relative to -940/GUS.

Discussion

Transformation by microprojectile bombardment has been successfully applied to *Arabidopsis* seedlings in the past (Bruce et al., 1989, Seki et al., 1991). In one case (Bruce et al.,1989), CAT was used as the reporter gene, and the enzyme was extracted from bombarded tissue for quantitation. Seki et al. (1991) report expression of GUS in transformed *Arabidopsis* seedlings from histochemical staining, but do not present a quantitative analysis of those activities.

Attempts were made in this study to quantify the expression levels that were observed in bombarded seedlings, but these were not successful due to high intrinsic fluorescence of the crude extract. Partial purification of the extract by chromatography may reduce background (Jefferson, 1987). It should also be instructive to segregate extracts with regard to tissue type, thereby minimizing the initial level of background fluorescence.

Microprojectile bombardment transformation may prove to be the method of choice for transient expression assay in *Arabidopsis*. Results obtained with this method compare well with those derived from transformed *Arabidopsis* protoplasts. Furthermore, analysis of gene regulation in the intact plant as opposed to undifferentiated tissue is

Refinement on the area of reporter gene expression quantification from intact plant organs will provide the investigator of gene activity levels with an attractive alternative to protoplast transformation.

When mature seedlings were transformed by particle bombardment, ectopic expression of *Adh* was observed. This may be the result of the ultimate location of the introduced gene not being within chromatin. It may be argued that all components necessary for *Adh* mediated GUS expression are present in the leaf, and that *Adh* in chromatin is repressed by its context. Further experimental controls must be designed to preclude this result as an artifact.

Silicon carbide fiber ("whisker") transformation does not generate the number of transformation events necessary for meaningful quantification of gene expression. Using the method described in this study, an impractical number of replicate samples would be required for strict statistical analysis. This method would be useful only if transformation efficiency were improved. Repeated attempts here and elsewhere (J. Baier, personal communication) have been unsuccessful in this regard.

Transformation of protoplasts provided the most quantitatively accurate measurement of gene activity. It was found, however, that incorporation of a reference gene construct is necessary for the reduction of standard

deviation to acceptable levels. The 35S/Luciferase construct worked well for this purpose, and may also be useful as a reporter gene system also.

Mutagenic disruption of GBF binding to the G-box decreases gene activity by greater than 60% when assayed in protoplasts. This result is reflected in the bombardment assay, where activity of these mutant constructs is reduced, but not eliminated, as compared to wild type. Although the GM1 and GM2 mutations disrupt GBF binding unequally, both result in a decrease in GBF binding affinity (Figure 3-2). Thus, the GBF/G-box interaction is essential for full reporter gene activity in transient expression.

Deletion of the full length promoter to -390 resulted in no decrease in gene activity. Further deletion to -289 reduces gene activity by approximately 50%, and gene activity is nearly abolished by deletion to -177 (>90% reduction). All regions which are shown by this method to be essential for full gene activity include sequence elements which footprint *in vivo* (Ferl R. J., and Laughner , B. H., 1989). Whether these footprinted elements are protein-DNA interactions necessary for gene expression is not, however, proven.

Although results obtained by particle bombardment are much less quantitative, they may be useful for comparison of the assignment of phenotype with a particular

expression level or construct. GUS activity is detectable with expression levels as low as 30% (GM1 and GM2), and marginal GUS expression of the -177/GUS construct (8% in protoplasts) was evidenced by infrequent, very diffuse and faint blue spots. Given the ease of this technique, it may be applied to the rapid screening of constructs for phenotype assignment.

The question of cell type specific expression, consequently, may not be addressed by particle bombardment. The *Adh* deletion and mutation constructs used in this study have been cloned into a binary vector for Agrobacterium mediated transformation of *Arabidopsis* (Appendix A). Transgenic plants may then be used to further examine the involvement of these 5' flanking sequences in regulating the developmental, tissue specific, and anaerobically induced expression of *Adh*.

CHAPTER 4
SUMMARY

In vivo DMS footprinting of the *Arabidopsis Adh* 5' flanking sequences (Ferl and Laughner, 1989) provided a starting point for the in vitro characterization of protein interactions at the G-box (described in Chapter 2), elements downstream at -190 and -170 (Delisle and Ferl, 1990), and the ARE like region at -140 (A. Delisle, 1991). Sites of protein-DNA interaction identified by the in vivo footprint spanned from -140 to -310 as shown in Figure 4-1. Functional analysis in protoplasts of the 940bp full length promoter and deletions thereof (Chapter 3) define a region from -390 to -77 within which reside all sequence elements necessary for full gene activity. One of those elements identified by footprinting has been altered within the full length promoter (-210) and found to be essential for full gene activity. Thus, in vivo DMS footprinting has proven to be a useful technique for the identification of regulatory protein-DNA interactions.

Adh 5' flanking sequences between -940 and -390 are dispensable for full transient gene activity (Chapter 3). Further deletion to -289 results in a decrease in activi-

Figure 4-1. *Arabidopsis Adh* 5' Flanking Sequence Summary.

A) Sequence from -500 to -270 is shown here with regions of *in vivo* footprinting shown as bold sequence. Sites of deletion constructs are indicated by outlined sequence numbering.

B) Continuation of sequence shown in A, from -260 to -30.

A

-500 -490 -480 -470 -460 -450 -440 -430

AAGTGACGGCCAAGAATACAATTAAGAGCCAATTAGTGATCTTTCATAACTTTAAAAATCTCACAAAAGTAGAAAAAAAA

TTCACTGCCGGTTCTTATGTTAATTCTCGGTTAATCACTAGAAAGTATTGAAATTTTTAGAGTGTTTTCATCTTTTTTTT

-420 -410 -400 -390 -380 -370 -360 -350

AATTCCAACTTGATGACCAAGAATAATACTATTAAAGAGCTATTTAAGATGAAACCGCCCGAAACCAAAAGCATTCGATG

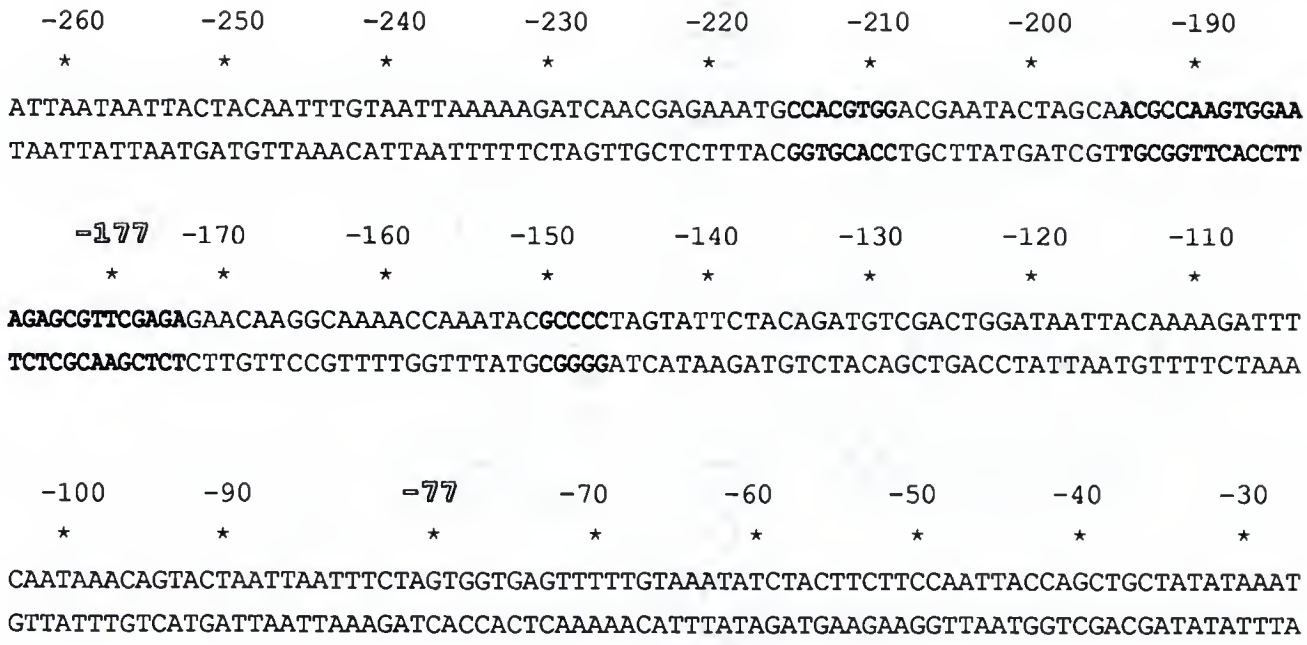
TTAAGGTTGAACTACTGGTTCTTATTATGATAATTTCTCGATAAATTCTACTTTGGCGGGCTTTGGTTTTCGTAAGCTAC

-340 -330 -320 -310 -300 -289 -280 -270

GGTACACCGATTACTGCTTTTAGCAACA**CCACGGCGTGA**CCATCAAGACTAATTAACTAAGACCACATTTTAAAAAAACT

CCATGTGGCTAATGACGAAAATCGTTGT**GGTGCCGCACT**GGTAGTTCTGATTAATTGATTCTGGTGTAAAATTTTTTTGA

B


-260 -250 -240 -230 -220 -210 -200 -190
ATTAATAATTACTACAATTTGTAATTAAAAAGATCAACGAGAAATGCCACGTGGACGAATACTAGCAACGCCAAGTGGAA
TAATTATTAATGATGTTAAACATTAATTTTTCTAGTTGCTCTTTACGGTGCACCTGCTTATGATCGTTGCGGTTCACCTT
-177 -170 -160 -150 -140 -130 -120 -110
AGAGCGTTCGAGAGAACAAGGCAAAACCAAATACGCCCCTAGTATTCTACAGATGTCGACTGGATAATTACAAAAGATTT
TCTCGCAAGCTCTCTTGTTCCGTTTTGGTTTATGCGGGGATCATAAGATGTCTACAGCTGACCTATTAATGTTTTCTAAA
-100 -90 -77 -70 -60 -50 -40 -30
CAATAAACAGTACTAATTAATTTCTAGTGGTGAGTTTTTGTAAATATCTACTTCTTCCAATTACCAGCTGCTATATAAAT
GTTATTTGTCATGATTAATTAAAGATCACCACTCAAAAACATTTATAGATGAAGAAGGTTAATGGTCGACGATATATTTA

ty of 50%. Examination of the in vivo DMS footprint in this region reveals only one site of protein binding at-310. This region has been subjected to limited characterization in vitro. An oligonucleotide was synthesized corresponding to the footprinted region at -310, and this was used in a gel retardation assay of protein binding competition with the -210 probe (Figure 2-4). The -310 element did not compete for -210 protein binding, and it was concluded that -310 does not bind the same protein(s) as -210.

Sequence of the footprint region at -310 is similar to -210, but not identical. The -310 domain contains a nearly symmetrical GTGG dyad which is hyphenated by a 3 bp insertion at the axis of symmetry. This sequence motif has not been explicitly characterized in other regulatory systems, but is similar to the Hex-1 type ASF-1 binding sites of the CaMV 35S promoter. Protein binding and competition analysis of the -310 element is necessary for initial characterization of this putative regulatory element.

Although only one in vivo footprint (-310) was observed within the -390 to -289 region, further expression analysis of this region is needed to verify its regulatory capacity. Mutagenesis or partial deletion of sequences comprising the -310 footprint (within the context of the full promoter) should be expected to

reduce transient expression. Provided that no other regulatory elements are present within the -390 to -289 region, or that their is no interaction between this and other regulatory elements of the promoter, the reduction in expression should be 50%, as was observed by deletion of the entire -390 to -289 fragment.

A similar mutational analysis was conducted for the G-box element in Chapter 3 (Figure 4-2). Disruption of protein-DNA interactions at this location reduced gene activity by 70%, whereas deletion of the G-box containing fragment reduced expression by an additional 40% (Zone B) beyond the 50% reduction associated with deletion to -289 (Zone A). Although these numbers are necessarily approximations, the possibility of disagreement should be examined.

Assuming that this observed discrepancy is accurate, the following possibilities are considered. First, there may exist an element within the -289 to -177 region which binds an inhibitory regulator, and whose binding is precluded by the presence of GBF. This may be partially evaluated by analysis of mutation of the -190 or -175 sequences in the full length promoter in the presence or absence of the G-box mutation. Mutation of G-box proximal inhibitory elements alone should have no effect on expression of the full length promoter. However, mutation of such putative elements in conjunction with the G-

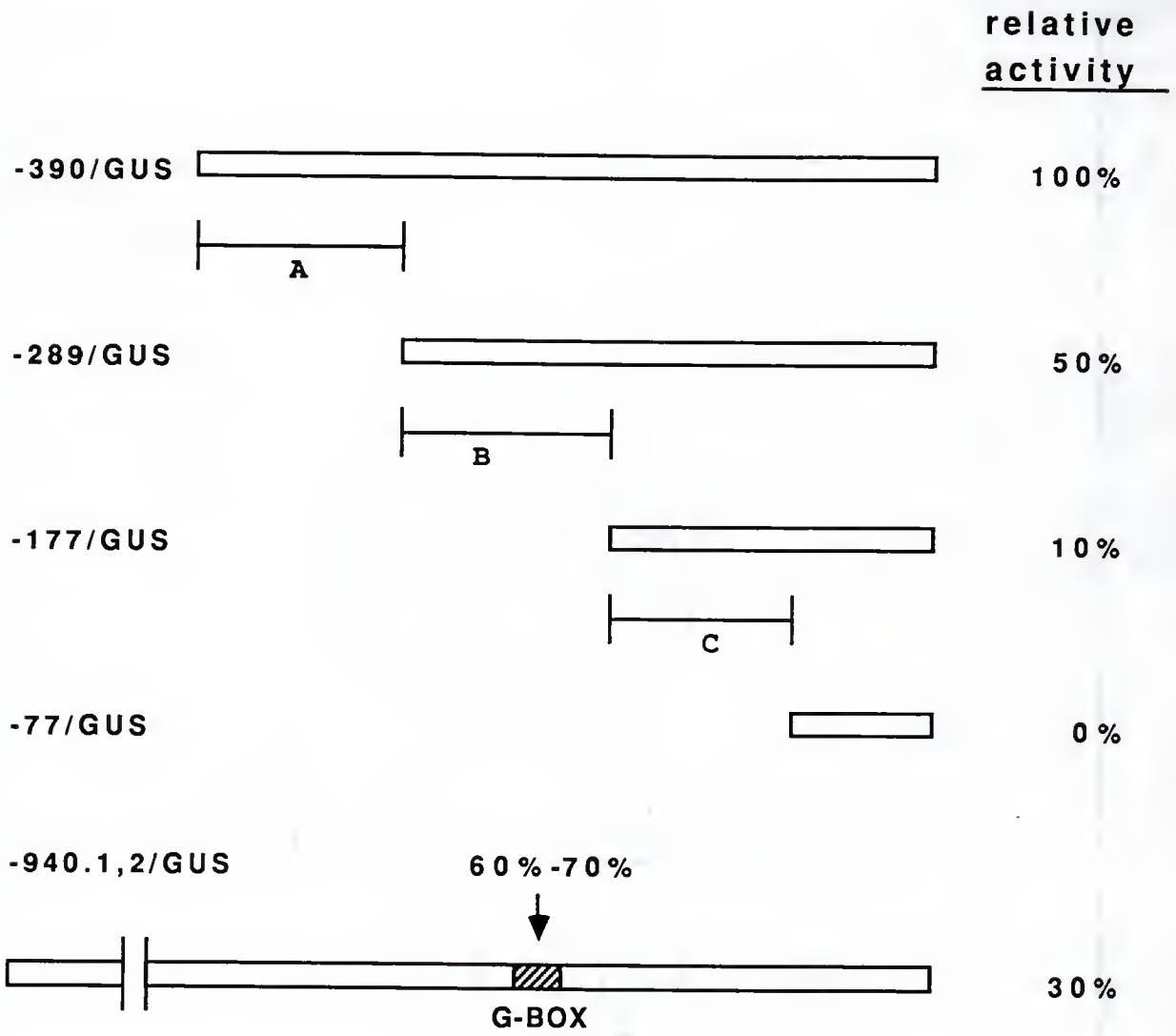


Figure 4-2. Summary of Relavent Protoplast Transformation Results.

Relative activities are approximations of data from Figures 3-35 and 3-36. Standard deviations ranged from 10% to 20%. Zones A, B, and C refer to the associated promoter fragment evaluated by 5' deletion.

box mutation(s) within the full length promoter should provide an activity level of at least 50% (from the -390 to -289 region), vs. 30% for -940.1 or -940.2.

The second possibility is that the G-box/GBF interaction functions synergistically in combination with an additional regulatory element, and its (G-box/GBF) deletion reduces activity to a level less than half of the activity contributed by both elements. This may be tested by evaluating the effect of mutation of particular candidate elements in conjunction with the G-box mutation. Putative interacting elements, when mutated in conjuction with the g-box mutation, should reduce activity to less than half of that level observed when the putative site is mutated alone.

Finally, deletion from -177 to -77 reduces gene activity from less than 10% to essentially zero. This fragment also contains two regions of in vivo DMS footprinting at roughly -170 and -145. This study suggests that the -177 to -77 fragment contains only those DNA elements necessary for assembly transcription complex, and expression from this truncated promoter is barely detectable by particle bombardment and histochemical staining. This result is supported by recent evidence that the -145 region binds specifically to a protein component of crude or partially purified *Arabidopsis* whole cell extract (Delisle, 1991). This protein or

complex of proteins was found by UV crosslinking to migrate between 300kd and 400kd. A 350kd molecular weight has also been reported for TFIID from Drosophila, and was found to be an aggregation of at least seven tightly bound proteins (Dynlacht et al., 1991). This complex is reported to be the target of upstream activating sequences in that system.

Current models of transcriptional activation by RNA polymerase II must take into account the ability of upstream regulatory elements to interact with and activate the transcription complex near transcription start. This difficulty is best handled by assuming that DNA may loop around, carrying any bound proteins with it, so that these activator proteins are brought within range of the target proteins of the polymerase (see review by Greenblatt, 1991).

A summary of this model, as it might pertain to *Arabidopsis Adh* is shown in Figure 4-3. More than one activation domain may interact with the polymerase complex, giving rise to a synergism of activation. Multiple activation domains would bring together polymerase subunits (such as TFIIB, and TFIID) thereby allowing for a more rapid assembly of the RNA polymerase II complex (Greenblatt, 1990). Furthermore, proteins which do not directly contact DNA may serve to bridge between DNA bound activators and the transcription complex.

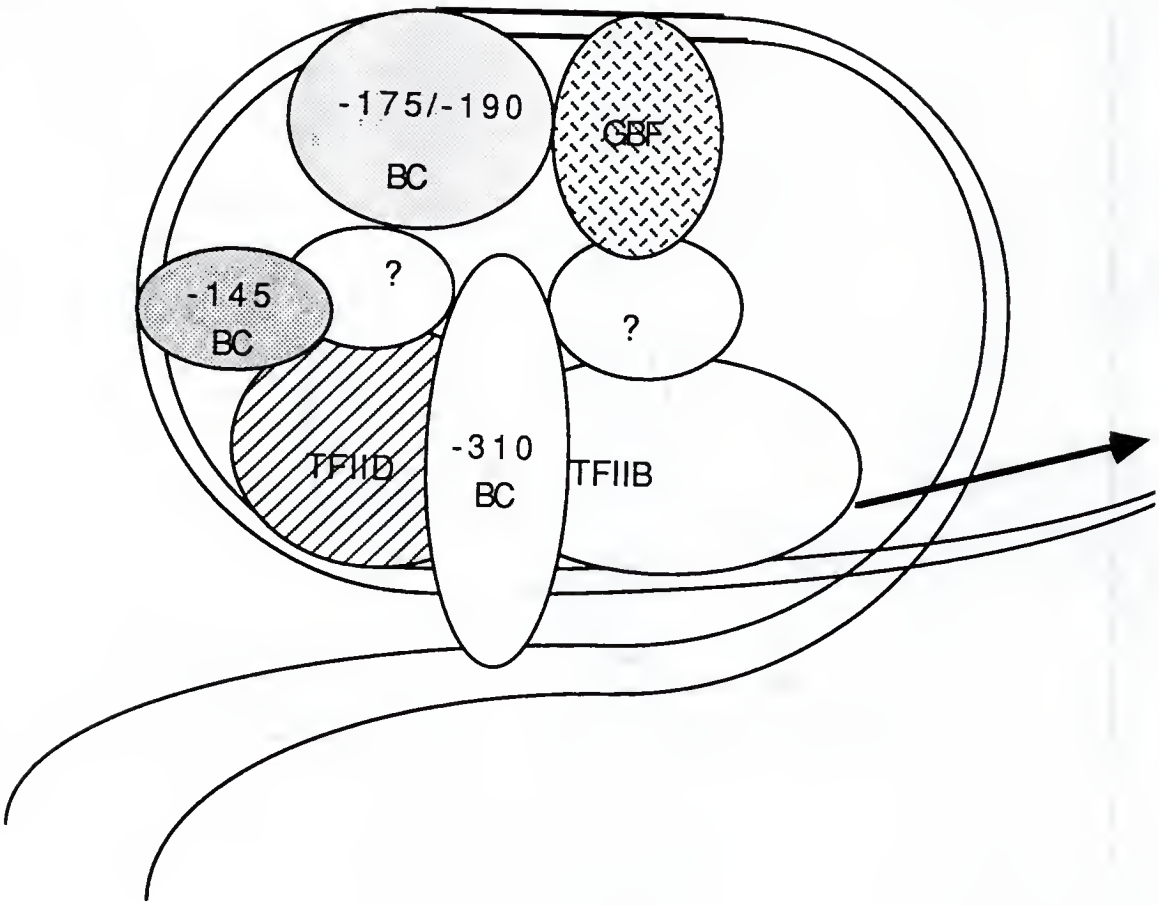


Figure 4-3. Model of *Arabidopsis Adh* Transcription Complex.

GBF indicates protein bound to the -210 region only. Other known components of RNA pol II transcription (TFIIA,E,F; TBF) have been ommited for clarity, and are not indentionally excluded.

The question arises how transcriptional activators are themselves regulated in their ability to activate the upstream regulatory sequences. Elements of the 5' flanking sequence of yeast *Adh* are bound by the transcriptional activator ADR1 (Cherry et al., 1989). ADR1 is binding of DNA is regulated by its state of phosphorylation. Phosphorylated ADR1 does not bind DNA, and *Adh2* in inactivated. ADR1 phosphorylation is regulated by a cyclic AMP-dependent protein kinase.

Phosphorylation has also been found to regulate binding of protein to photoregulated genes (Datta and Cashmore, 1989). The sequences to which this protein binds are AT rich, however, and this type of regulatory element has not been identified for *Arabidopsis Adh*. This may be in part due to the inherant limitations of in vivo DMS footprinting, which identify only those protein contacts at G residues. AT rich domains are present in the *Arabidopsis Adh* promoter within regions which this study has defined to be necessary for full gene activity (-230 to -270).

Of all the putative regulatory elements identified in *Arabidopsis Adh*, the G-box has received the most attention by far. This element has been identified and characterized as to function in a number of plant gene families. Also, proteins which bind to G-box elements have been cloned, and nucleic acid and protein sequence

data is available for EmBP-1 (Guiltinan et al., 1990). This protein is an activator of the wheat Lea (late embryo abundant) gene, *Em*, and will confer transient expression on a reporter gene in the presence of abscisic acid. The sequence element to which it is bound is therefore called ABRE (for abscissic acid response element). This ABRE is virtually identical to the *Adh* and other plant G-box elements, as well as the Hex elements of CaMV, Nos, and histone gene promoters (Katagari et al., 1989; Mikami et al., 1989) other G-box elements. These genes, as well as plant *Adh*, however, are not known to be activated by ABA (Marcotte et al., 1989; An et al., 1990), and this suggests that many types of G-box binding proteins may exist in the plant.

In fact, recent work by Meier and Grusseim (1991) has identified and cloned three separate G-box binding protein genes from tomato fruit. These genes are expressed only in fruit, and are not expressed in cotyledons or leaf tissue. This result suggests that the difference observed in Chapter 2 (Figure 2-4) between migration rates of protein/G-box complexes from cell cultures and leaves may also arise from binding of two different GBFs.

The results of this study may be summarized in the following proposed model for *Arabidopsis Adh* transcriptional activation. Assembly of the initiation complex

(Figure 4-1) is limited by availability of either transcriptional activators at -310, -210/-190. These activators are brought into proximity of the polymerase by DNA looping. Binding of the polymerase subunits extends from -145 to transcription start (Figure 4-1, A), or the -145 element binds an additional activator protein.

Tissue specificity may be regulated by availability of -310 or -210/190 activators or co-activating components, or the presence of a leaf specific inhibitor. The results presented here do not exclude the possibility of inhibitor binding within the -289 to -177 fragment. Analysis of the constructs examined here (Figure 3-3) in transgenic plants will provide tissue specificity results, and will allow the examination of induction by anoxia as well. Additional mutations and deletions suggested earlier in this summary may be introduced into *Arabidopsis* as well, allowing for a thorough comparison of transient and stable expression.

As work continues on the formation and activation of the RNA polymerase II complex, these results should be tested in models of plant mRNA transcriptional activation. Surely, mRNA transcription must not differ greatly among genes utilizing RNAP II. The question remains whether the factors influencing the rate of assembly of this protein complex are within the grasp of current understanding. A working model is needed for the ability

of the nucleus to choreograph the activities of all genes within the cell, as determined by the cells position, age, and role in the intact plant.

APPENDIX A
PREPARATION FOR ARABIDOPSIS STABLE TRANSFORMATION

The various deletion and mutation constructs of the *Arabidopsis* Adh 5' flanking sequence (Figure 3-7) have also been prepared for stable transformation into *Arabidopsis*. This was begun by first cloning the full length promoter (-940), the G-box mutant promoters (-940.1 and -940.2) and the promoter deletions into a suitable vector. The method selected for this purpose employs a binary vector (Bevan, 1984) which , when present in the appropriate *Agrobacterium* strain, will allow selected DNA sequences to be transferred into the plant (Zambryski, 1988).

Binary vectors are relatively complex and large (>10 kb) plasmid molecules designed to accomplish several transformation functions. First, a reporter gene must be present and preceded by a multiple cloning site. Promoter fragments are cloned into this region for subsequent assay of their regulatory properties in the transgenic plant.

The reporter gene system that we have selected is the Beta-glucuronidase (GUS) system described by Jeffer-

son (1987) which may be assayed histochemically in *Arabidopsis* tissues. GUS activity may also be extracted and assayed biochemically (Jefferson, 1987).

The binary vector must also include a selectable marker. This vector (pBI101)contains the neomycin phosphotransferase (NPT)/nopaline synthase chimaeric construct (Bevan et al., 1983). This gene confers resistance to kanamycin on the bacterium or plant in which it resides. Transformants will appear normal (green) on selective media, and those shoots which do not contain the transferred DNA will be yellow or brown.

The selectable marker gene and the reporter gene are flanked by the left and right borders of transfer DNA (t-DNA). T-DNA is that region of the *Agrobacterium* Ti plasmid which is transferred into the plant. These border elements are recognized by gene products of the host *Agrobacterium*, and direct the excision of t-DNA from the binary vector.

I will now describe the methods which I have used to clone the previously described Adh fragments into pBI101. These subclones were then used to transform *Agrobacterium* strain LBA 4404. Transformed *Agrobacterium* will then be used for transformation of *Arabidopsis*.

METHODS

DNA Constructs

Deletion fragments were originally constructed to possess a 5' SstI site and a 3' BamHI site for directional cloning into a CAT expression vector. The CAT vector used for our previous experiments contained the pUC multiple cloning region in an orientation such that an SstI/BamHI cut promoter fragment will insert in the correct orientation. This SstI site was used for constructing the GUS expression vector by cloning the GUS coding sequence into pUC multiple cloning site, however, so the vector pBI101 contains an SstI site between the GUS coding region and the nos terminator sequence.

The selected deletion fragment is then cloned into the binary vector as follows. Plasmid pBI101 is first linearized by digestion with SalI and BamHI. The linear vector is then included in a ligation which contains the desired insert (which has been cut with SstI and BamHI) and a single stranded "bridge" oligonucleotide. This oligonucleotide was designed so that the overhanging ends of SalI and SstI digests may be annealed in a blunt ended manner. This method was successful for subcloning, but destroyed both SalI and SstI sites in the final construct.

The pBI101/Adh chimaeric constructs were cloned into E. coli strain HB 101. A recombination$^-$ strain was necessary to prevent rearrangement of the introduced construct. Purified plasmid DNA was then used for transformation into *Agrobacterium* strain (Clonetech).

Agrobacterium transformation

The *Agrobacterium* strain LBA 4404 was transformed by the following method (M. Ashraf, personal communication). Fifty ml of LB media containing 50 ug/ml Streptomycin was inoculated with *Agrobacterium* from a glycerol stock, grown for 3 days at 28^0C, 125 rpm. On the third day of growth, 5 ml of *Agrobacterium* culture was transferred to 50 ml LB/Strep, and grown at 28^0C, 135 rpm, until reaching an A_{600} of 0.3. The culture was then stored on ice.

Cells were harvested by centrifugation at 5000 rpm, 4^0C, for 5 min., and resuspended in 10 ml of cold 0.15 M NaCl. The cells were then spun again at 5000 rpm, 4^0C, for 5 min., and resuspended in 1.0 ml of cold 20 mM $CaCl_2$. Cells were then transferred in 0.2 ml aliquots to 1.5 ml eppendorf tubes to which 200 ug of the appropriate DNA was added. The mixture was stored on ice for 30 min., and then frozen in liquid nitrogen for 1.0 min.. The mixture was then allowed to thaw at 37^0C, after which 1.0 ml of LB was added. Cells were allowed to recover at

28^{0}C for 2 hr. with gentle shaking, and subsequently recovered by a 1.0 min. spin in a microcentrifuge. The pellet was resuspended in 100 ul LB and plated on streptomycin + kanamycin at 50 ug/ml each. Colonies appeared in 2 to 3 days and were screened for the presence of the binary vector by the method of Hooykas (1988). Transformed *Agrobacterium* was stored as a glycerol stock at -80 until use for *Arabidopsis* root explant cocultivation.

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BIOGRAPHICAL SKETCH

William Lee Mckendree, Jr. was born in 1954 in Miami, Florida. After graduation from high school in 1972, he attended Okaloosa-Walton Jr. College until 1974 and then transferred to the University of Florida, which he attended until 1977. He then enlisted in the US Navy where he served until 1981. Following honorable discharge, he then returned to the University of Florida where he completed a BS in botany in 1982. Continuing at the University of Florida, he completed an MS in botany in 1985, and a Ph.D. in botany in 1991.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Ferl, Chairman
Professor of Botany

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Thomas E. Humphreys
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Larkin C. Hannah
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William B. Gurley
Associate Professor of Microbiology
and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry S. Nick

Harry S. Nick
Assistant Professor of Biochemistry
and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the Department of Botany in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree Doctor of Philosophy.

December, 1991

Dean, Graduate School